2017

Polyanydride Nanoparticles: A Drug delivery vehicle to kill intracellular pathogens

Paul Lueth

POLYANYDRIDE NANOPARTICLES: A DRUG DELIVERY VEHICLE TO KILL INTRACELLULAR PATHOGENS

PAUL LUETH

DEPARTMENT OF VETERINARY MICROBIOLOGY AND PREVENTIVE MEDICINE

IN PARTIAL FULFILLMENT OF NON-THESIS MASTERS DEGREE

INTERDISCIPLINARY GRADUATE STUDIES PROGRAM

JULY 14, 2015

Overview

- Introduction to IGS
  - VMPM
  - IMBIO
  - CHEM E
- Project Outline
- Key Observations, Results, Conclusion
- Future Plans

Interdisciplinary Graduate Studies Program

DESCRIPTION

Available to graduate students who wish to have a more diversified program of advanced study than generally permitted for students who specialize in a single subject

Program is open to any qualified graduate student, who wish to improve their subject matter competence in more than one discipline.

COURSEWORK

Allowed to take courses in three different graduate subject matter areas

Each subject contributing a **minimum** of nine (9) semester credits toward the 35 semester graduate credits required for the degree

NON-THESIS OPTION

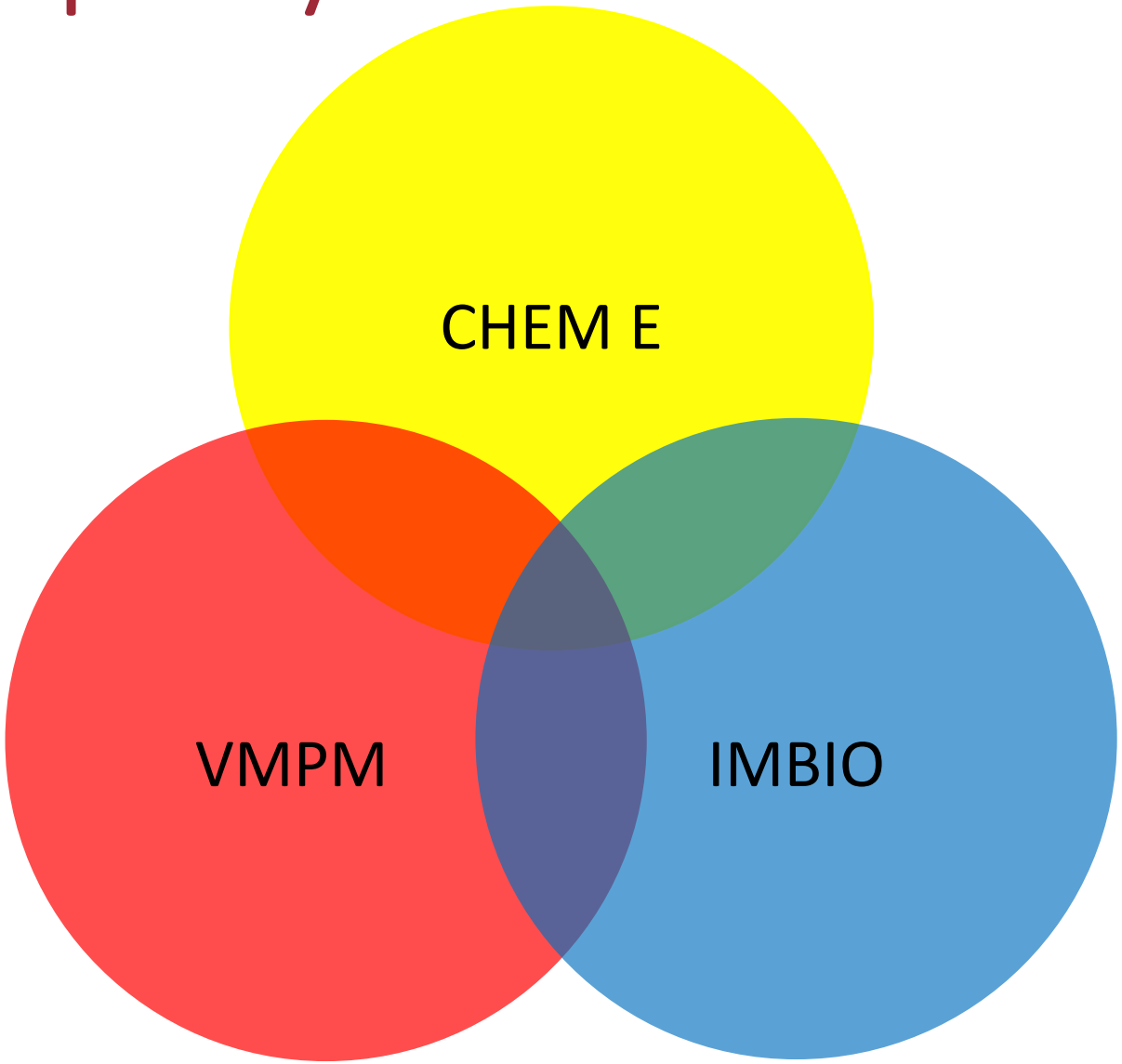
A creative component is required in which the student demonstrates independent creativity such as a written report of laboratory, field or library research, a project in fine arts, or some other original contribution.

Interdisciplinary Graduate Studies Program

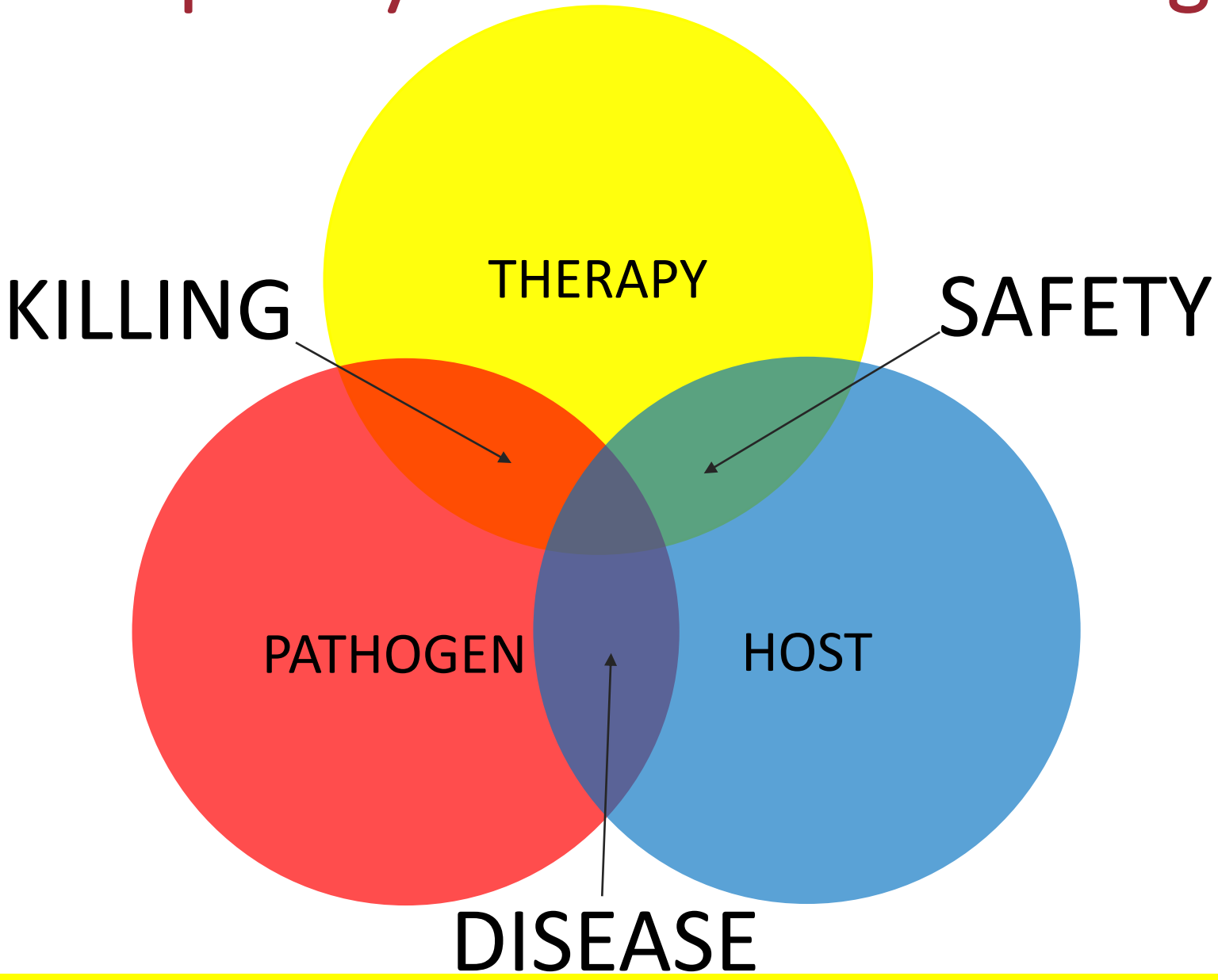



The student, in consultation with his/her Program of Study Committee, will decide on the choice of option (i.e., thesis or non-thesis). The Program of Study Committee also aids the student in planning a program of study, selecting appropriate courses, and determining foreign language requirements, if applicable.

Interdisciplinary Graduate Studies Program



Interdisciplinary Graduate Studies Program



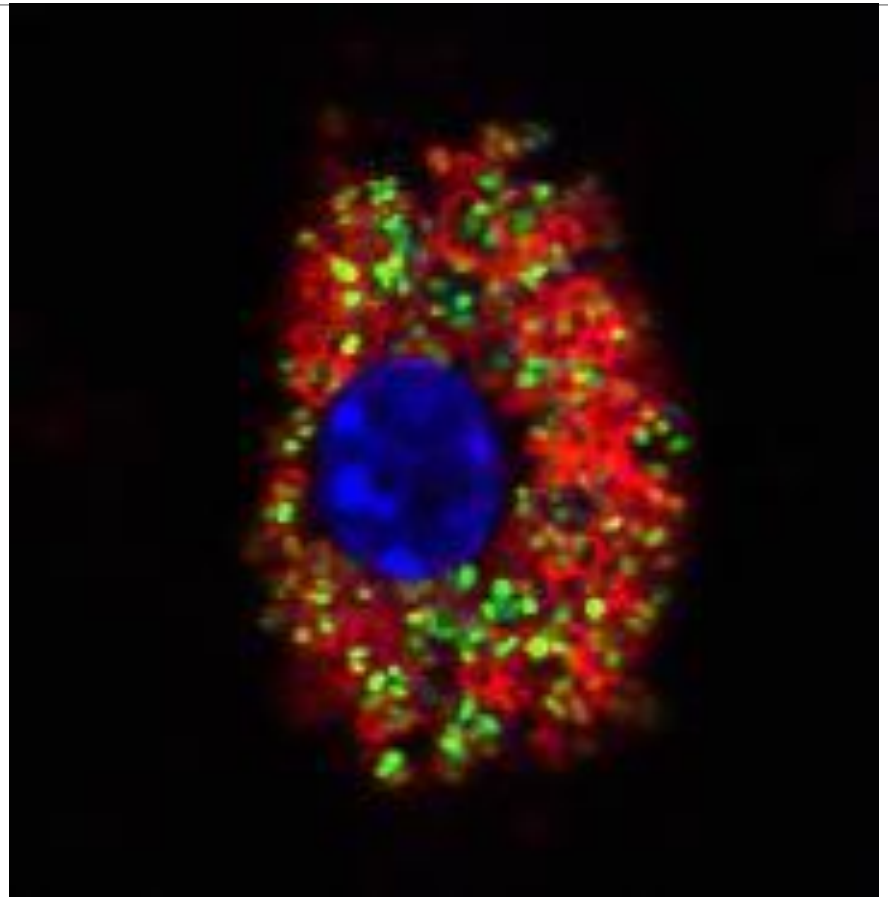
Brucellae

Gram Negative

Coccobacillus

Faculative Intracelluar Pathogens:

10 Species



Species

Species	Biovar/Seravar	Natural Host	Human Pathogen
B. melitensis	species	Goat, sheep	yes
B. abortus	1-6,9	Cattle, bison, buffalo	yes
B. suis	1, 2, 3	Swine	yes
	4	European hares	Yes
	5	Reindeer, caribou	yes
B. canis	none	Dogs, other canids	yes
B. ovis	none	Sheep	no
B. neotomae	none	Rodents	no
B. maris		Marine mammals	Yes(?)
B. pinnipediae			
B. cetaceae(?)			

Human Transmission

Rarely Fatal

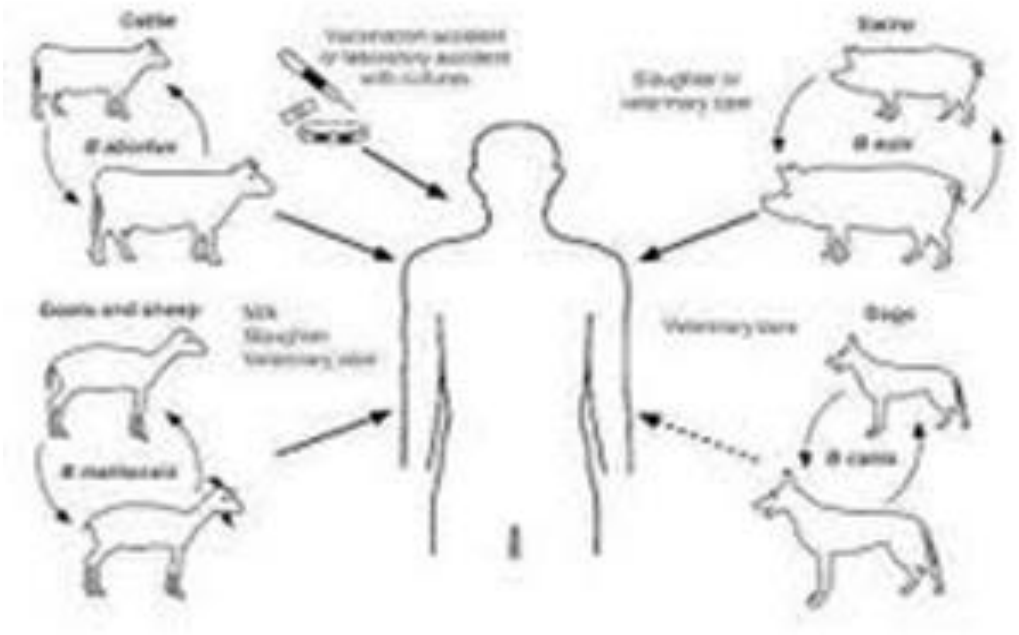
Not transmissible from person to person

Zoonotic

Highly infection through aerosol

10-100 bacteria for infection

Conjuctiva, skin lesions, contact with infectious tissue, ingestion of unpasteurized dairy



http://www.vet.uga.edu/vpp/archives/ivm/ENG/zoonoses/domestic/images/brucella_img02.jpg

Brucellae

Symptoms in Humans

Undulating Fever

Headache

Anorexia

Complications include endocarditis, meningitis, arthritis

Brucella

Treatement

Daily Combination of Doxycycline & Rifampin

Regiment 6-8 weeks

Relapse

No Vaccine for Human Brucellosis

Prevention

- Animal Vaccines
- Avoid direct contact with visibly sick animals
- Wear eye protection and gloves when handling carcasses
- Do not drink unpasteurized milk

Brucellae

Biological Threat

CDC Category B Biological Threat Agent

- Debilitating Symptoms
- Significant Morbidity
- Relapse of Infection
- Lack of Human Vaccine

Brucellae

Virulence Factors

Intracellular nice

LPS very little endotoxicity & poor inducer of cytotoxic mediators

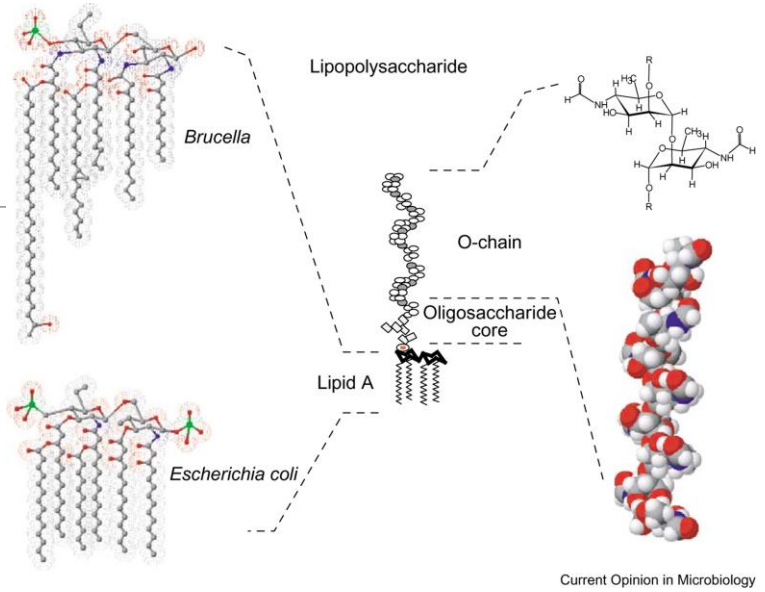
Smooth O-chain are resistant to serum & complement, enter via lipid rafts

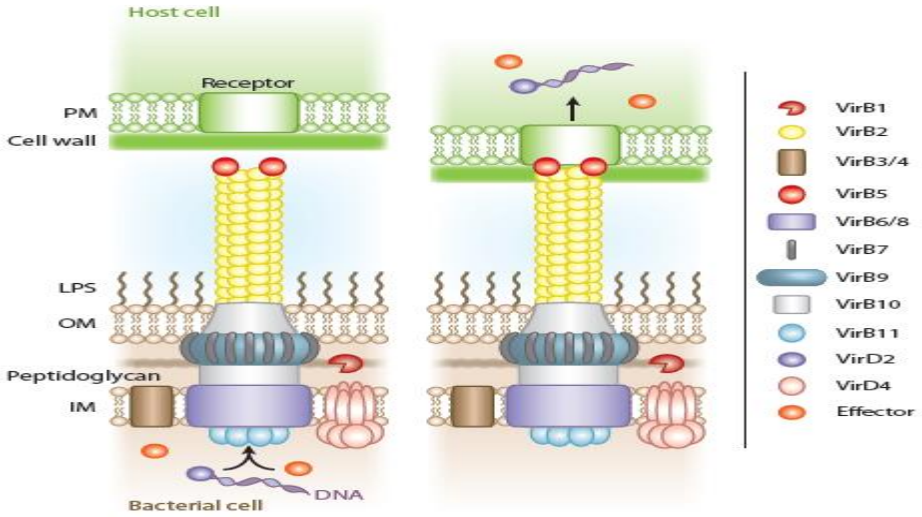
Type IV Secretion System

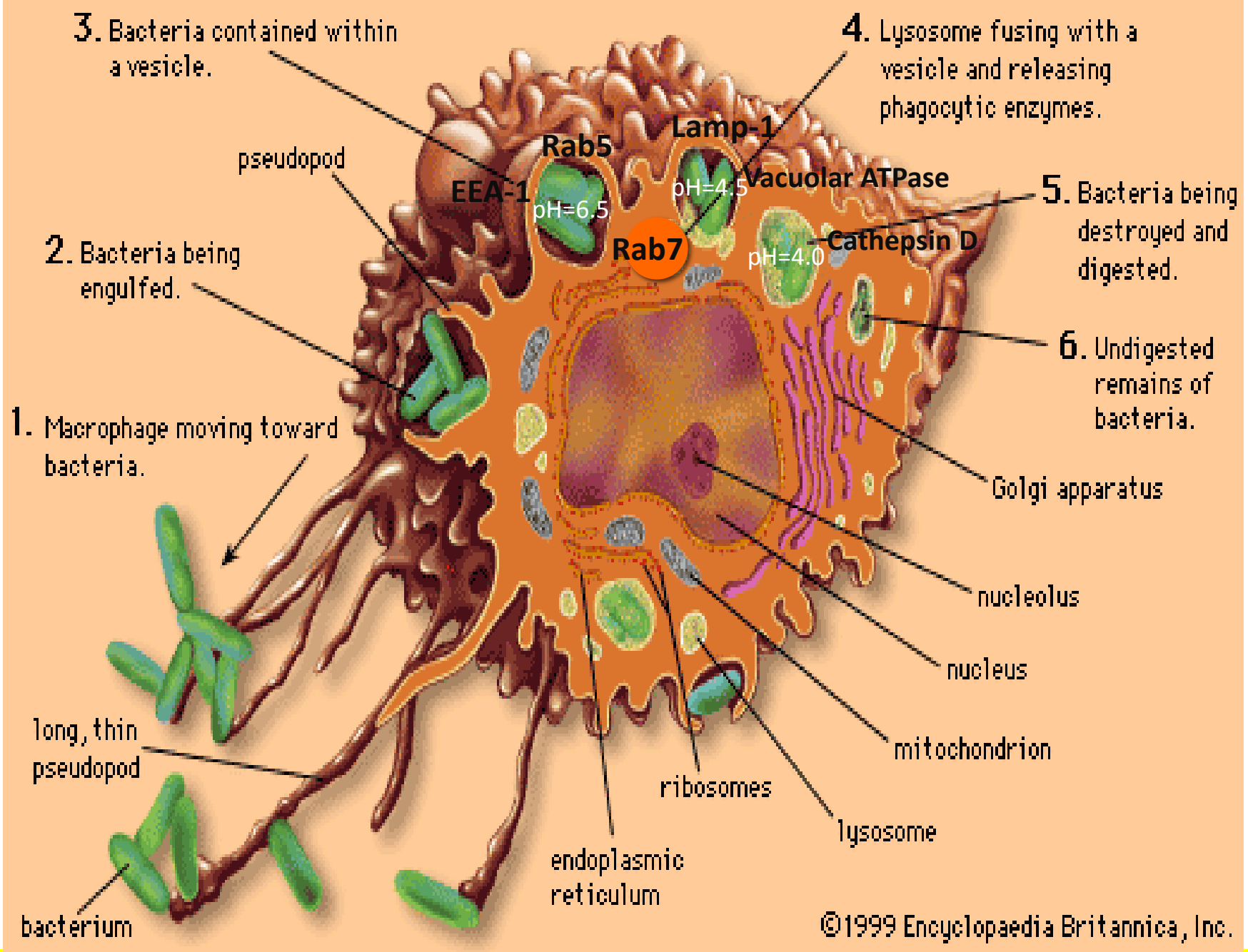
Cyclic β-1,2-glucans

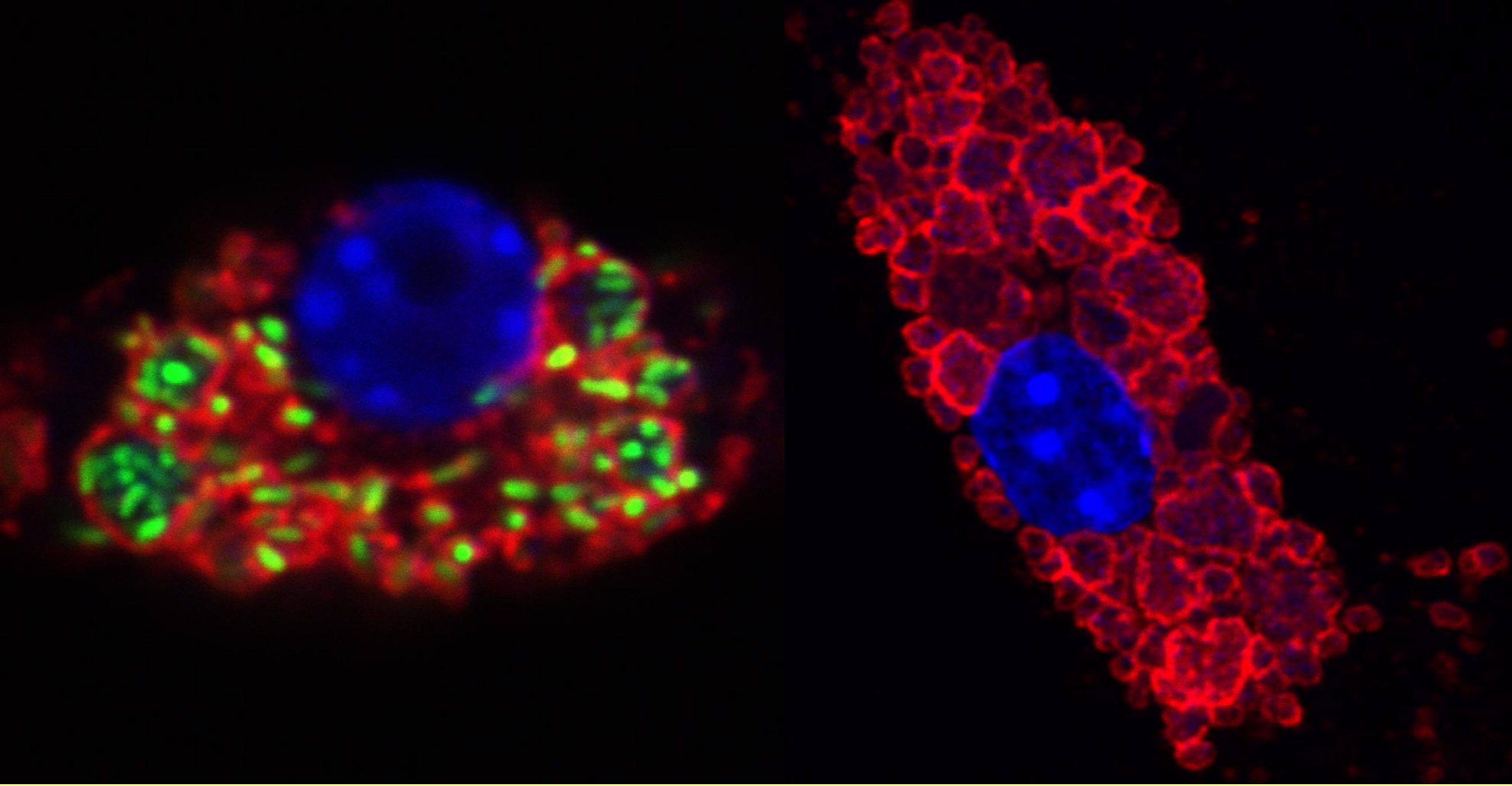
Prevents Apoptosis

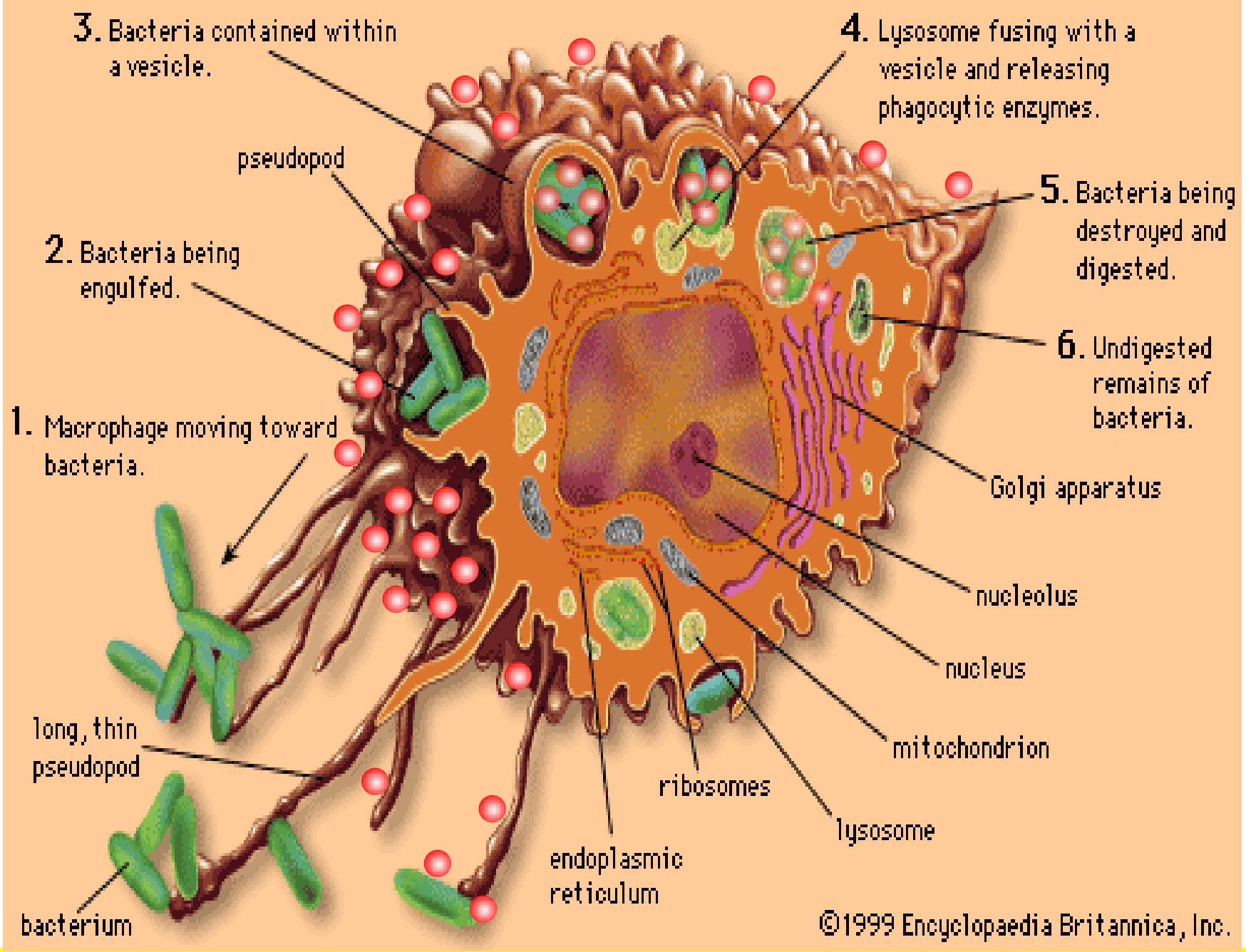
(2,3-DHBA) & 2,3-DHBA-based siderophore brucebactin (Bellaire et al, 2003).






3. Bacteria contained within a vesicle.
4. Lysosome fusing with a vesicle and releasing phagocytic enzymes.
Rab5
Lamp-1
pseudopod
EEA-1
pH=6.5
pH=4.5
Vacuolar ATPase
5. Bacteria being destroyed and digested.
Rab7
Cathepsin D
pH=4.0
2. Bacteria being engulfed.
6. Undigested remains of bacteria.
1. Macrophage moving toward bacteria.
Golgi apparatus
nucleolus
nucleus
mitochondrion
long, thin pseudopod
ribosomes
lysosome
endoplasmic reticulum
bacterium
©1999 Encyclopaedia Britannica, Inc.

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Brucella Host Cellular Immune Response

Effective Response requires CMI

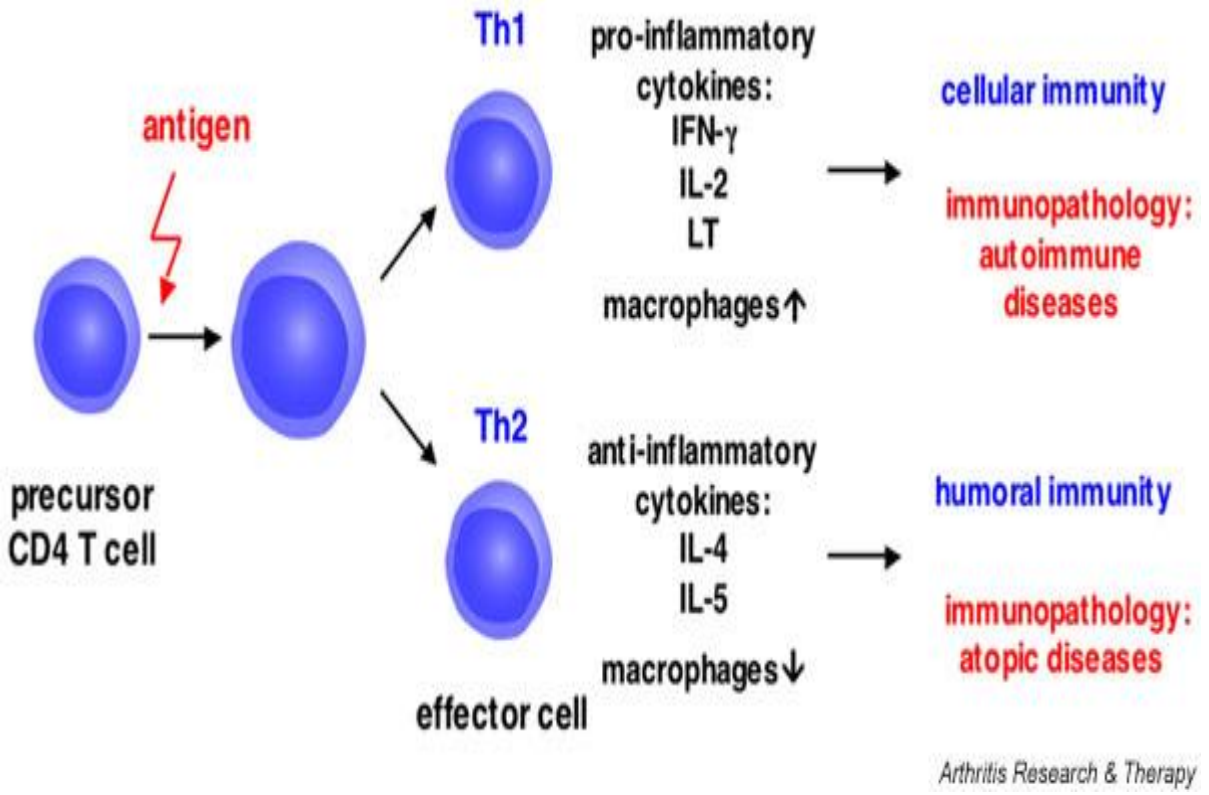
Cell-Mediated Response

- T_H1 response
- Activate Macrophages

Immunity is based on IFN-γ

- Controlled by IL-12 and TNF-α

ROI & NO contribute to Control

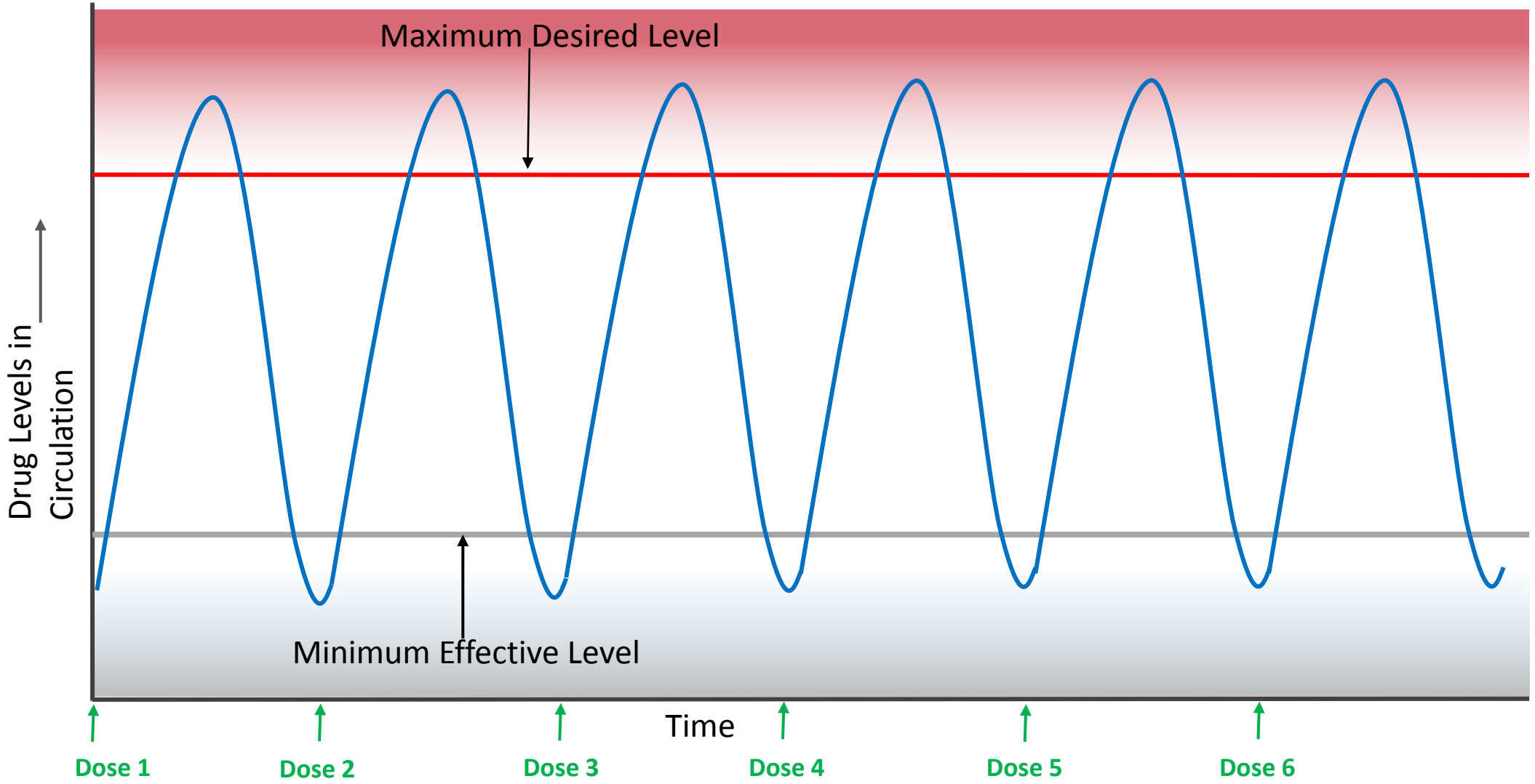


Drug Delivery

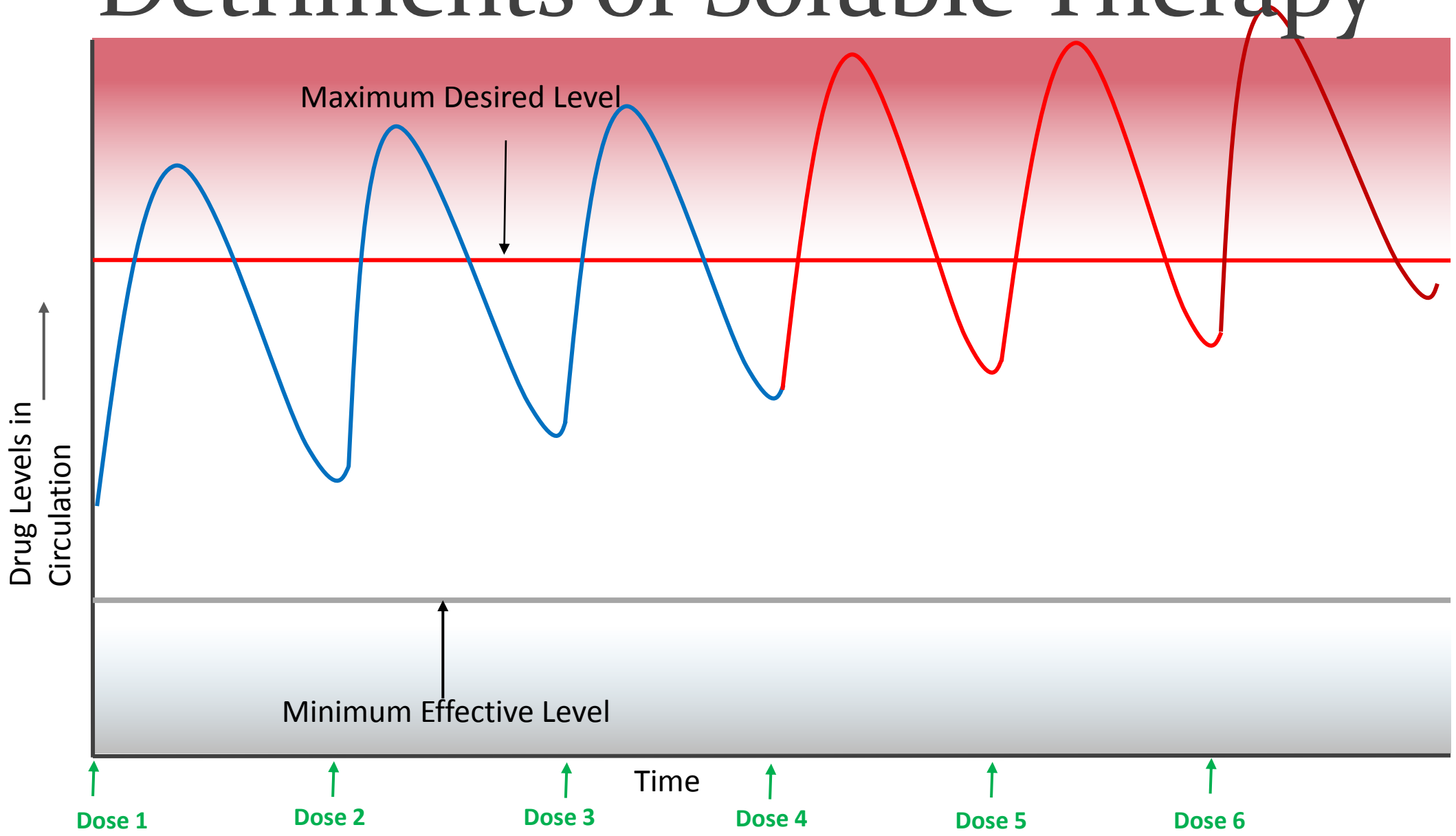
"refers to approaches, formulations, technologies, and systems for transporting a pharmaceutical compound in the body as needed to safely achieve its desired therapeutic effect."

http://www.reference.md/files/D016/mD016503.html

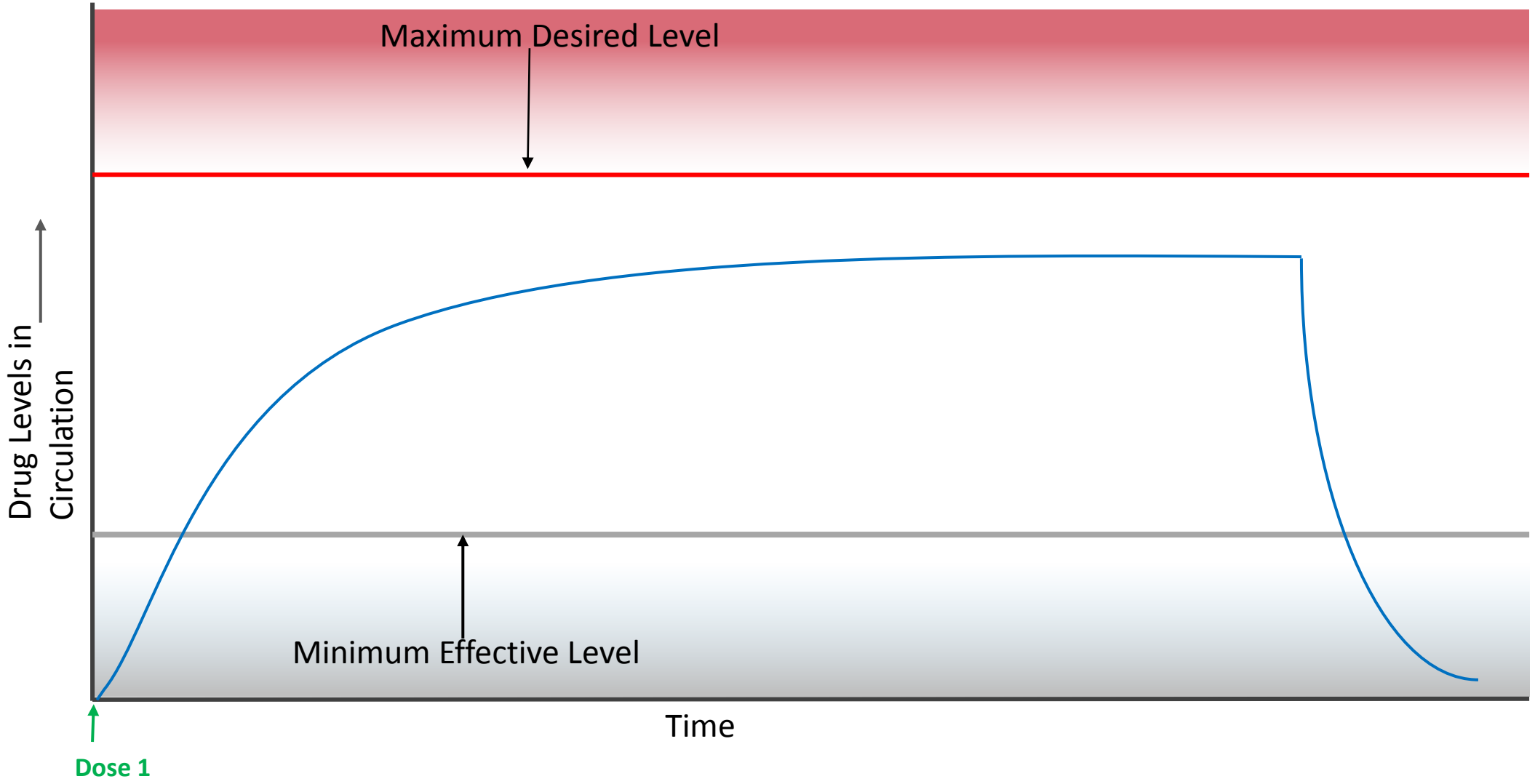
Therapeutic Index



Detriments of Soluble Therapy



Controlled Drug Delivery



Drug Delivery

Must stabilize drug

- Drug must be bioactive.
- Therapeutic drugs (proteins), prone to deactivation (synthesis)
- Proteins, in soluble form, may lose their functional properties (in vivo)

Administer drug at a concentration

- necessitates therapy
- precludes toxicity
- Drug to be released in a controlled, and sustained manner.

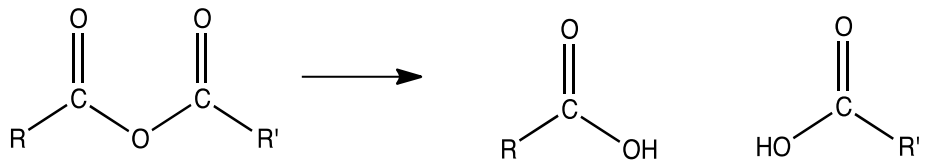
Material that encapsulates and delivers drug should be biodegradable

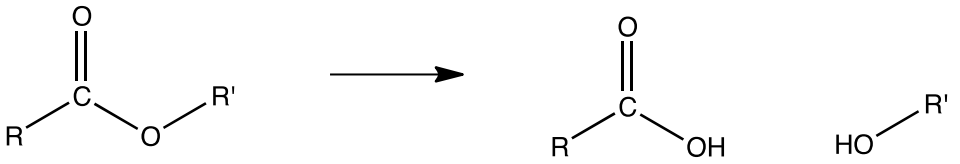
Biodegradable Polymers

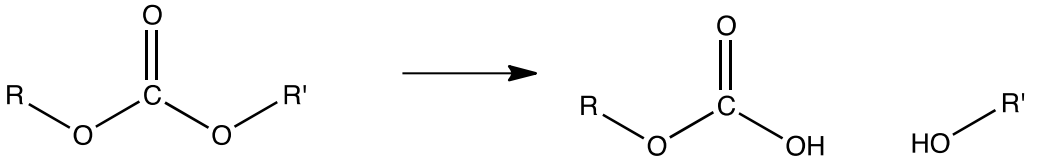
- Encapsulates drug
- Safely degrade
- Excellent candidates for controlled release
- Versatility in degradation mechanism
- Two main classes: Polyanhydrides and Polyesters
- Two degradation mechanism

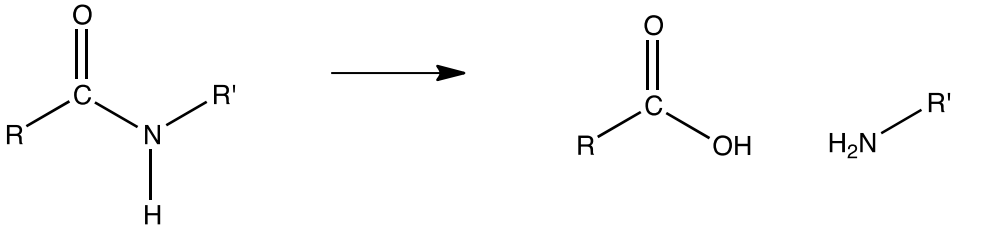
What can be hydrolyzed?

1. Anhydrides
2. Esters
3. Carbonates
4. Amide









Fast

Slow

Polyesters vs. Polyanhydrides

Both approved for use in humans

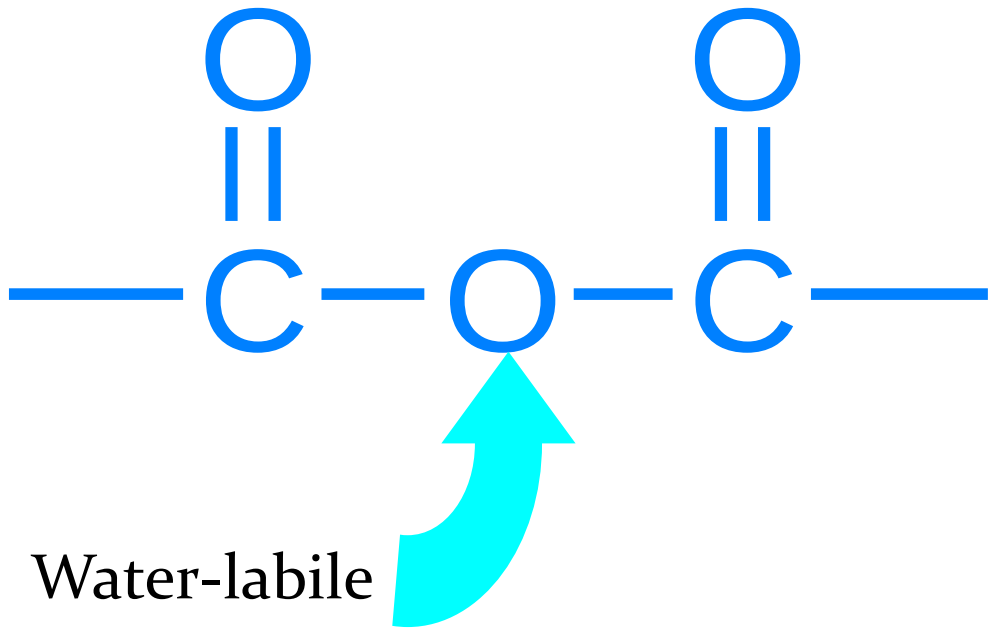
Both have tailorable release rates

Polyesters: bulk eroding, acidic degredation products

Polyanhydrides: surface eroding, very hydrophobic

Polyanhydrides

- Anhydride bond
  - Hydrolytically unstable
  - Degrades into two carboxylic acid groups
- Suitable for drug delivery systems
  - Gliadel®
- Surface erodible



Domb, Amselem, Langer, and Maniar, in *Biomedical Polymers Designed-to-Degrade Systems* (1993)

Commercialized Controlled Release Formulations

Brand Name	Application	Release time	Delivery route
GLIADEL® WAFER (polifeprosan 20 with carmustine implant)	Anti-tumor	Weeks	Implant
Once-a-day Claritin-D® 24 Hour (10 mg loratadine/240 mg pseudoephedrine sulfate, USP) Extended Release Tablets	Allergy	1 Day	Oral
Lunelle™ monthly contraceptive injection	Contraceptive	1 Month	Injection
PROZAC® Weekly™ fluoxetine hydrochloride	Anti-depressant	1 Week	Oral

Bulk Erosion

Modified
1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG)

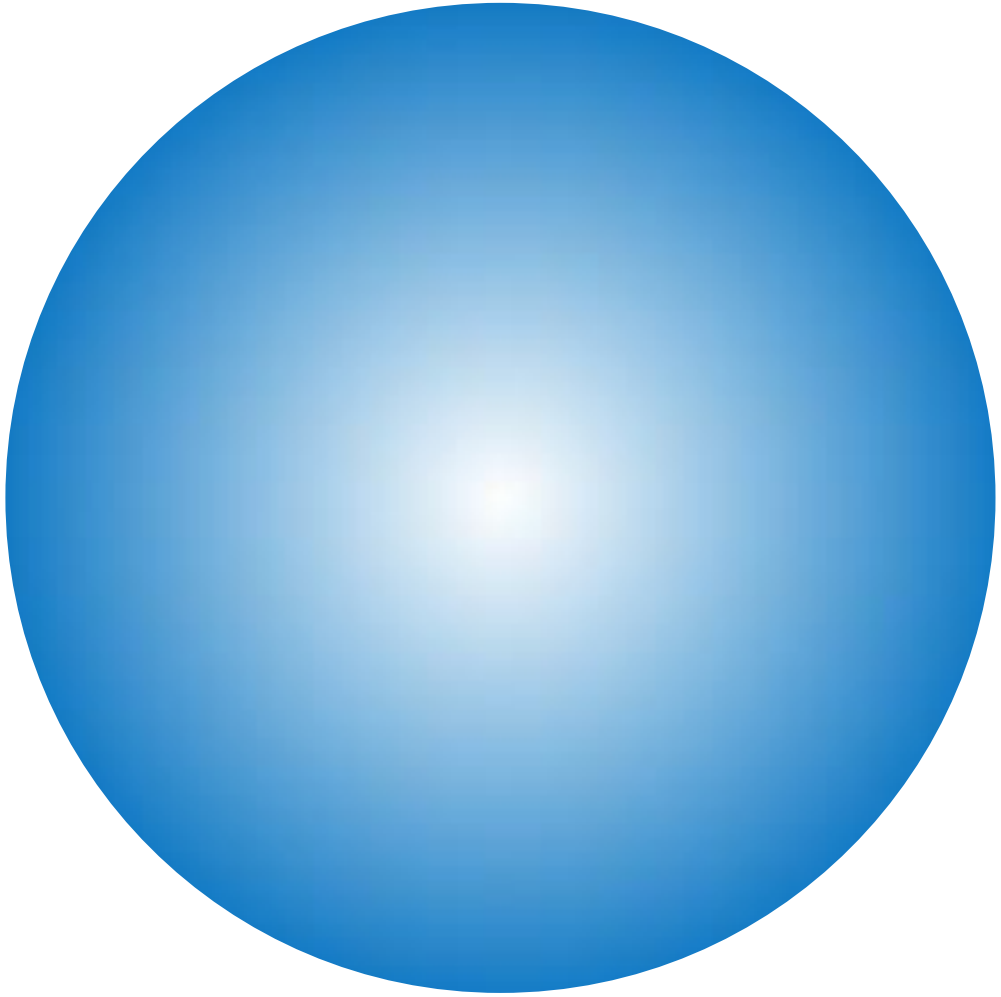
Hydrophilic backbone

Porous

Allow water to diffuse through surface

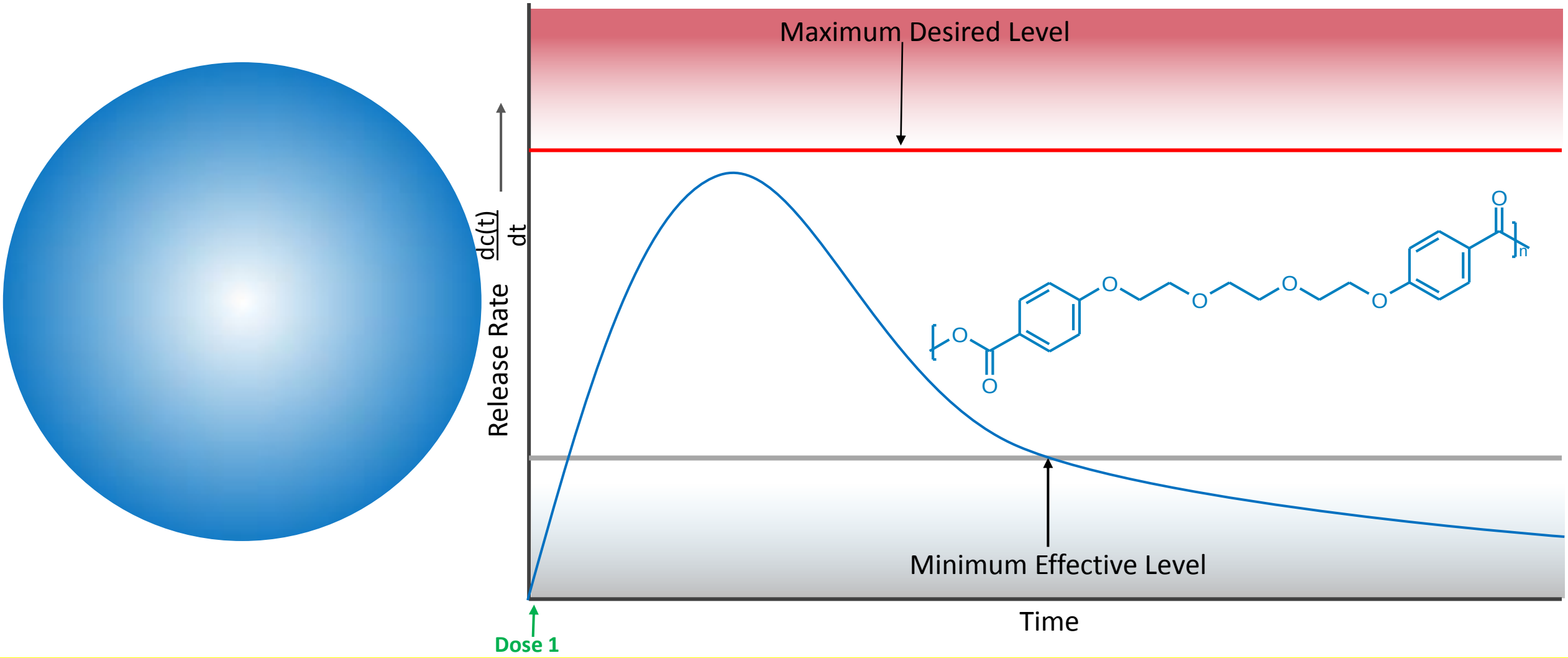
High porosity

Fast process



Modified

1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG)



Surface Erosion

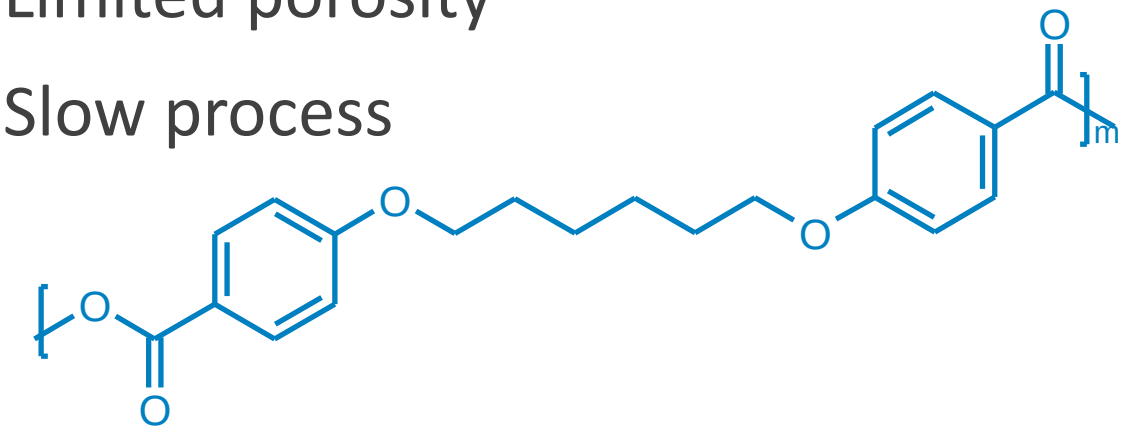
Surface Erosion

Hydrophobic polymers

Prevent water from penetrating interior

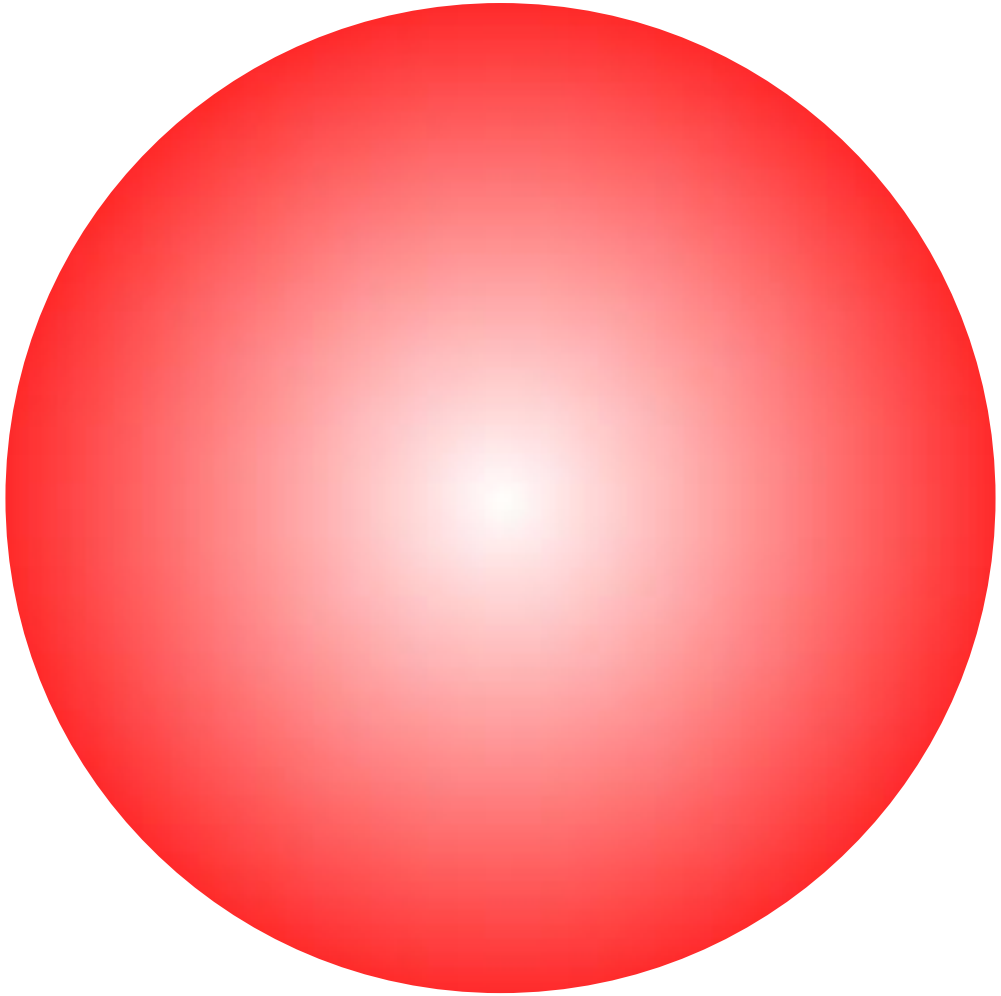
Limited porosity

Slow process



Hydrophobic anhydride monomer

1,6-bis(*p*-carboxyphenoxy) hexane (CPH)



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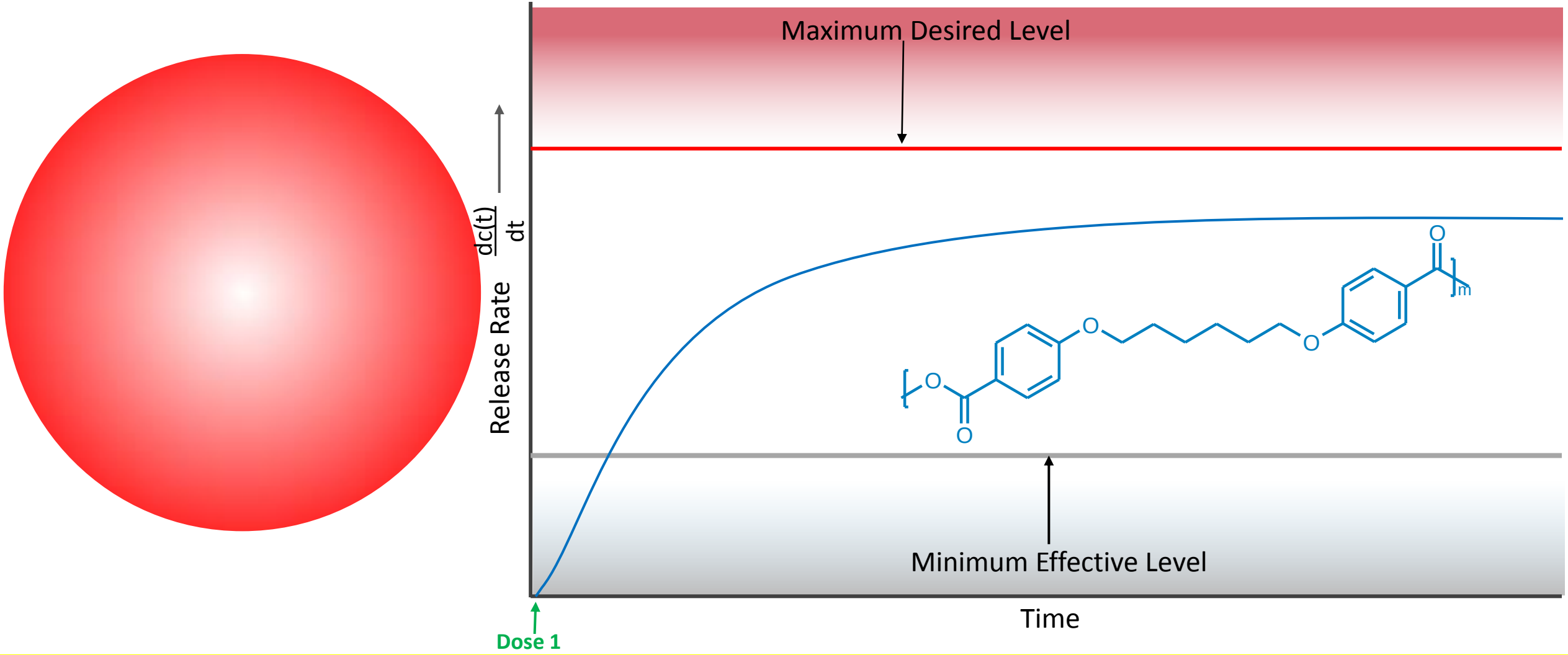
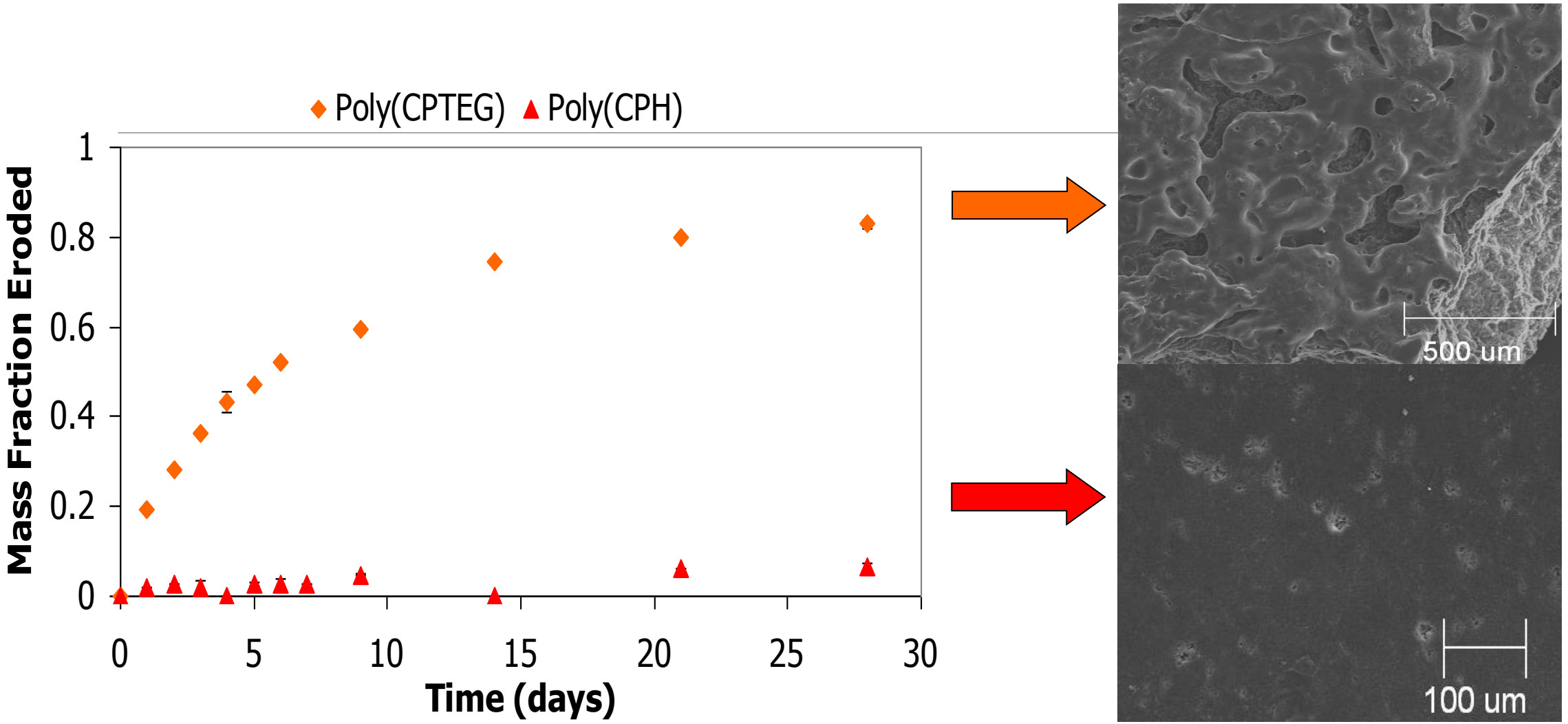


Erosion Mechanism of CPTEG:CPH



Polymers exhibit distinct erosion profiles that can be controlled by tailoring copolymer composition

Torres, Vogel, Mallapragada, and Narasimhan, *J. Biomed. Mater. Res.* (2006)

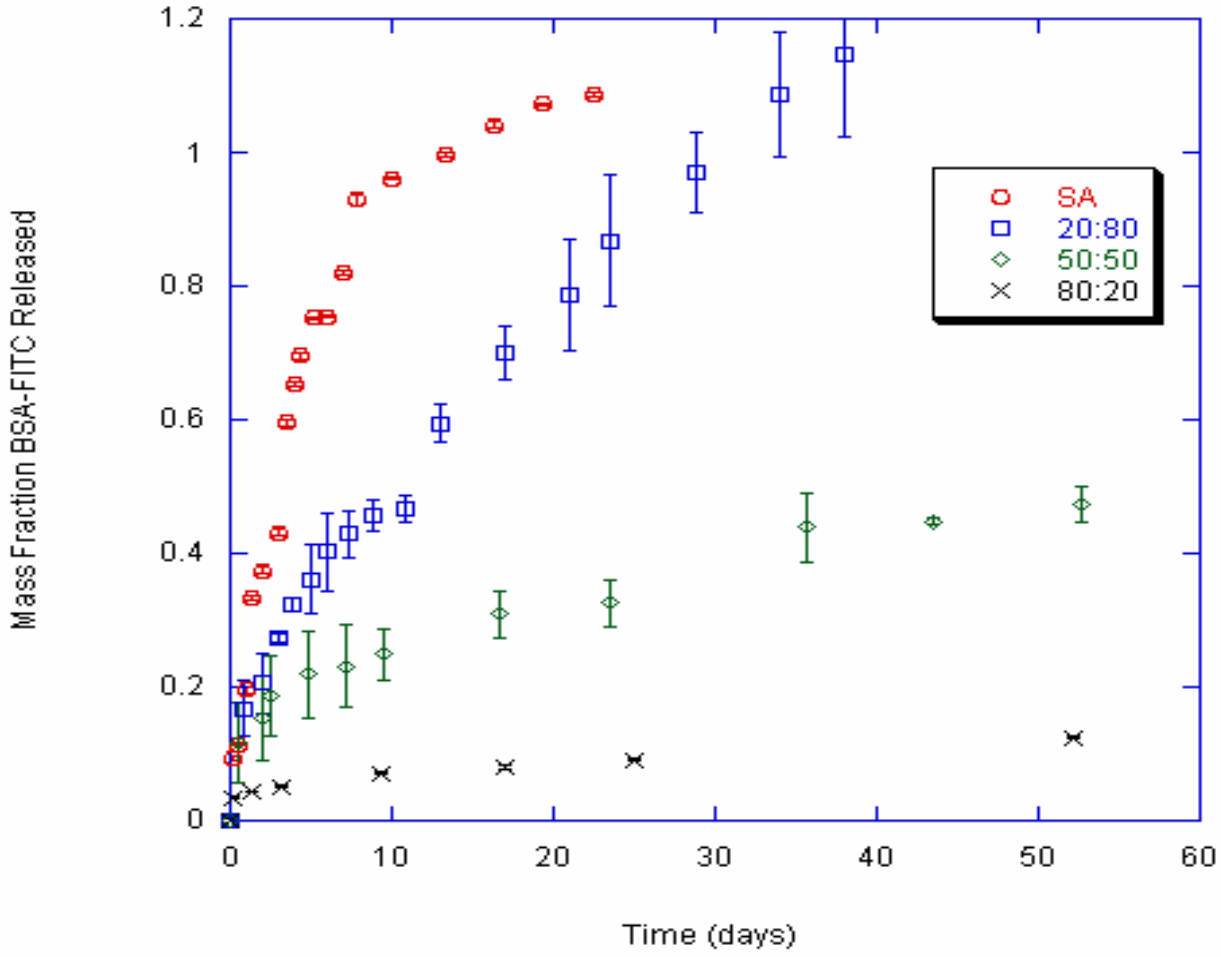
Chemistry Matters…

Polymer degradation
- Bulk erosion (e.g., CPTEG)
- Surface erosion (CPH & SA)

Drug/protein release

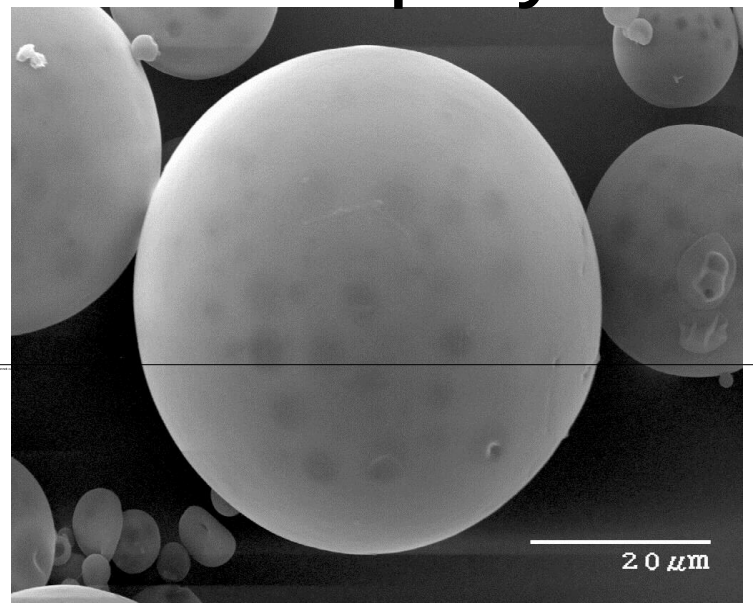
Protein/antigen stabilization
- Structure
- Function

Immunomodulatory vaccines
- Single dose
- Efficacious immune responses

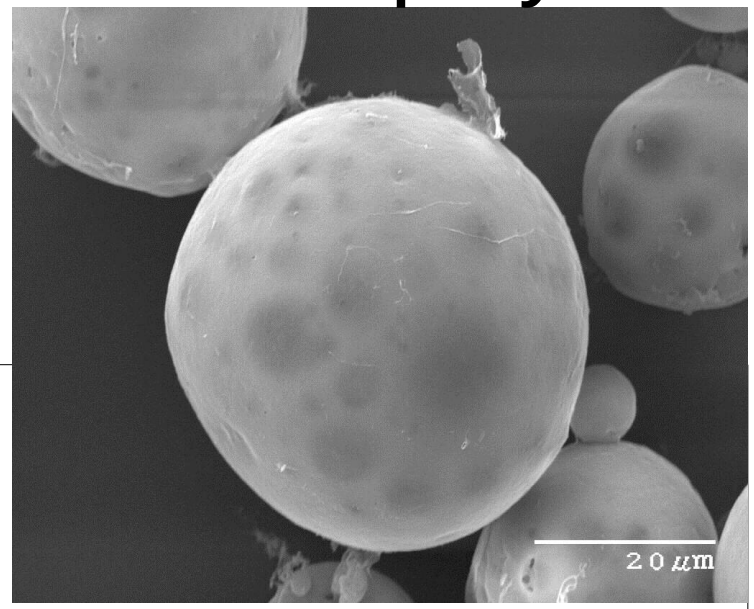


Shen et al, *J. Contr. Rel.* (2002) Determan et al, *J. Contr. Rel.* (2004) Kipper et al, *J. Biomed. Mater. Res.* (2006)c

20:80 copolymer



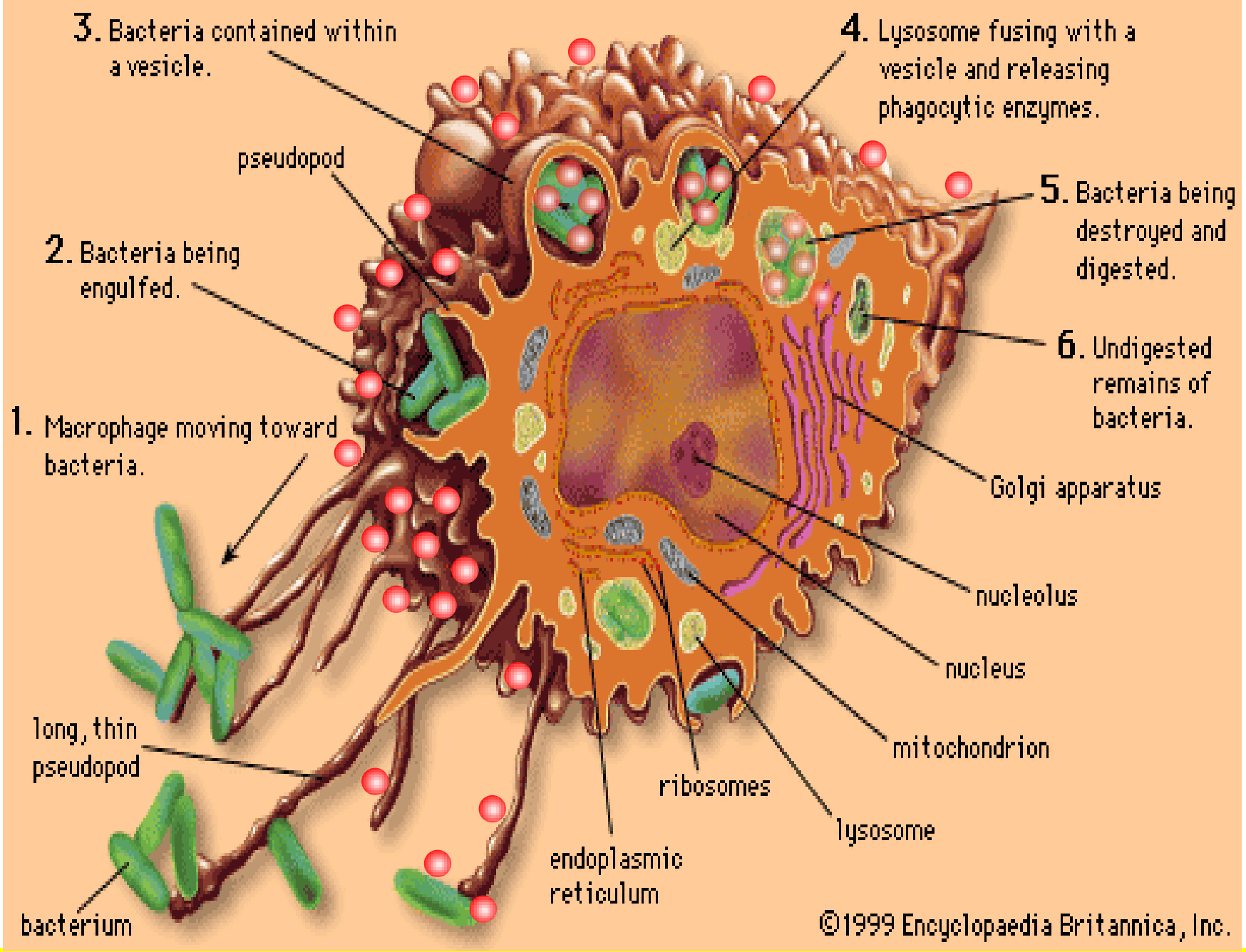
50:50 copolymer



Wilson Weldter et al 2006

Project Goal

Develop and assess polyanhydride nanoparticles as an intracellular antibiotic delivery vehicle.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Golgi apparatus
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bacterium
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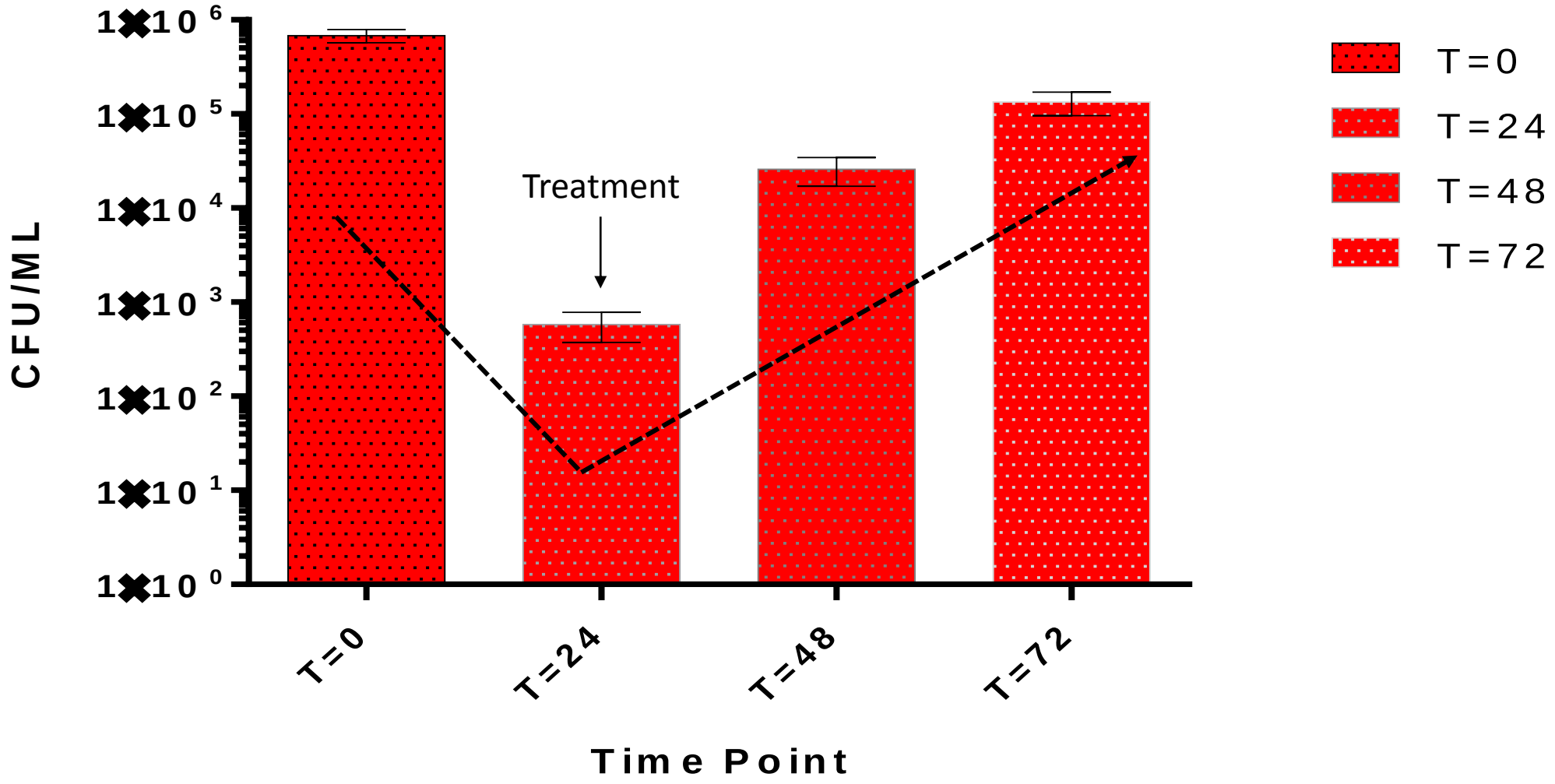
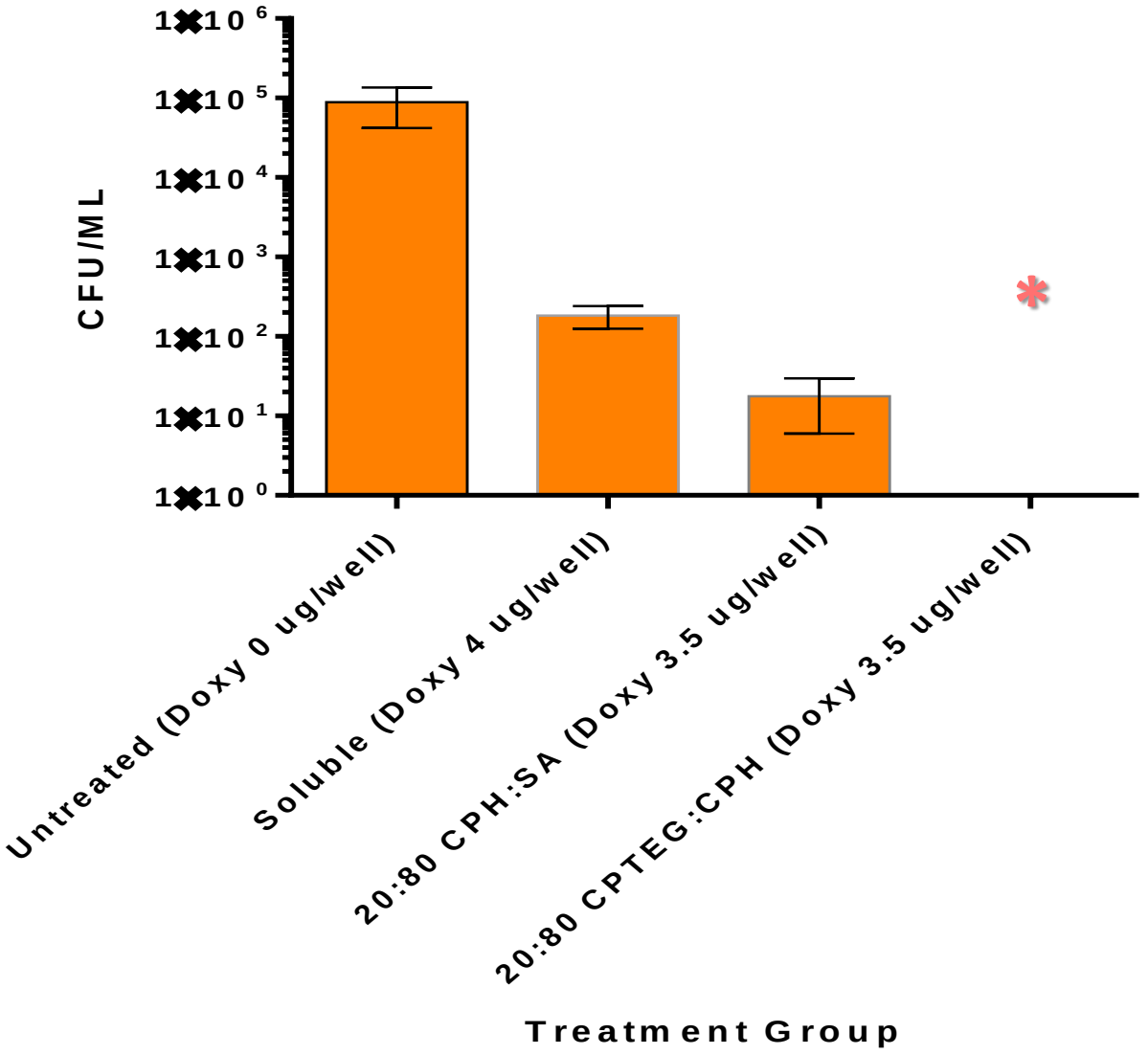
Time course of *B. melitensis* in Raw Cells



Intracellular Efficacy of PA nanospheres encapsulated w/22% Doxycycline, against *B. melitensis* in RAWS, 48 Hrs Post-treatment



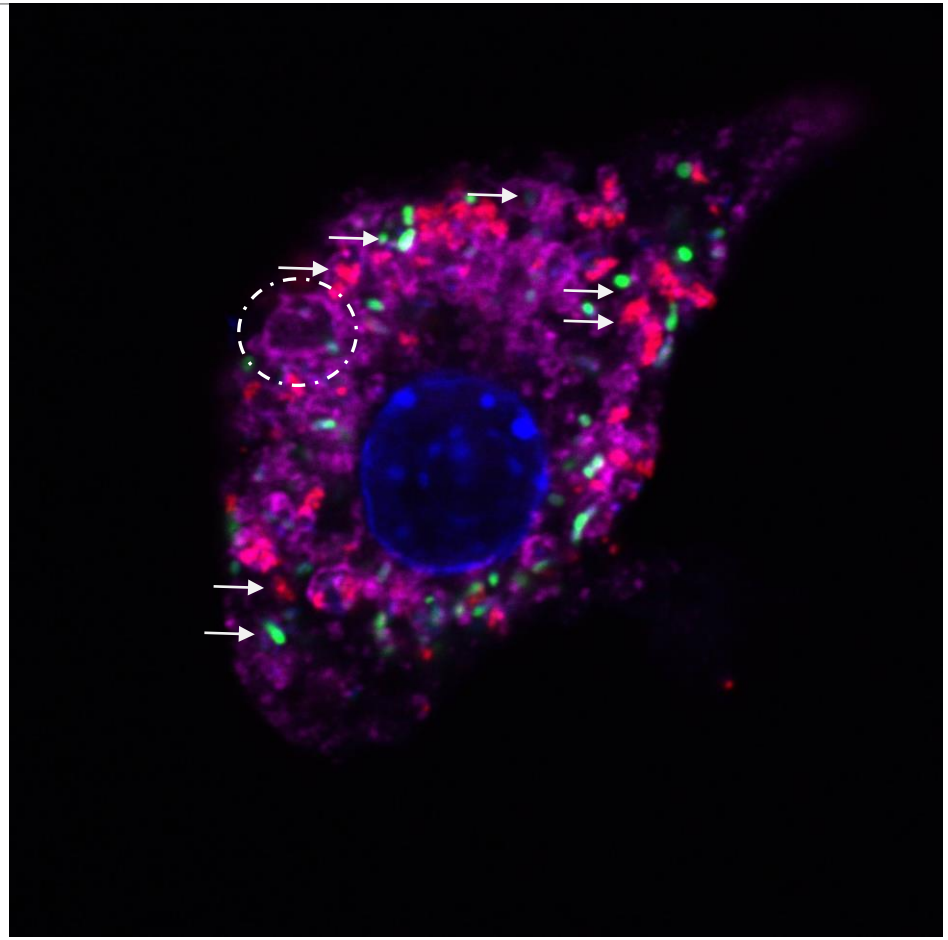
* $p < 0.05$c

Results

- Encapsulated PA nanoparticles were effective
  - Depot
  - Controlled release
  - Reduced/eliminated bacterial load
- 20:80 CPH:SA more effective after 24HRS
- 20:80 CPTEG:CPH more effective after 48 HRS, with no viable CFU

Key Observation

- At later time points, the number of cells containing nanoparticles decreased with infected cells
- Within the phagosome, there was limited colocalization between nanoparticles and *Brucella*.
- Nanoparticles and/or *Brucella* were not always localized within the phagosome



Incorporate encapsulated & blank nanoparticles in In vitro *Brucella* killing assay.

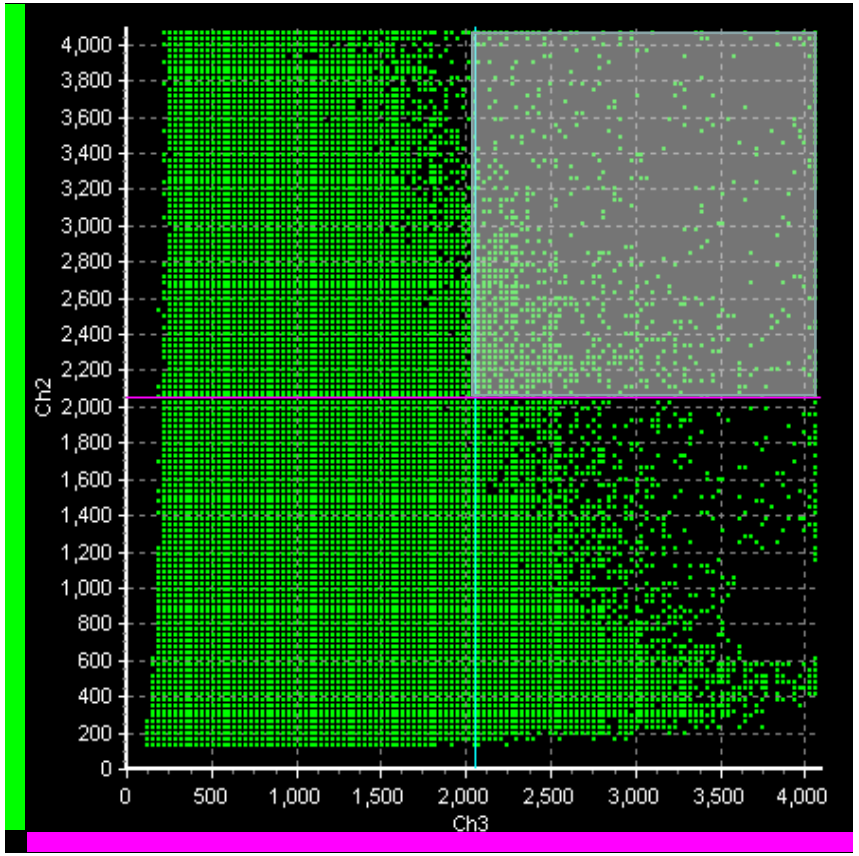
Hypothesis

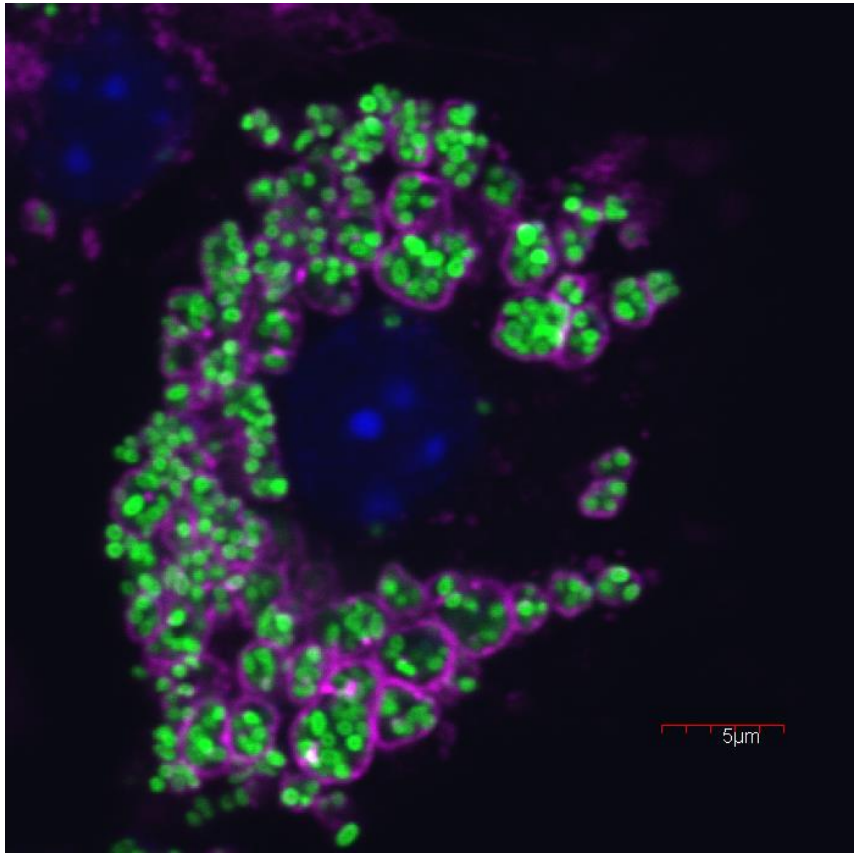
1. Colocalization between *Brucella* and NPs (antibiotics + dye), would not occur, as the NP arrive at their depot and releasing antibiotic, which kills *Brucella*, overtime and eliminating any GFP fluorescence.

2. Colocalization between *Brucella* and NP (dye alone), would occur, as the NPs arrive at their depot but do not contain antibiotic. Allowing *Brucella* to survive and fluoresce GFP.

Brucella melitensis in RAW 264.7 CELLS, 72 HRS P.I.

Colocalization of *B. melitensis* *(GFP)* and LAMP-1 *(Cy5)*

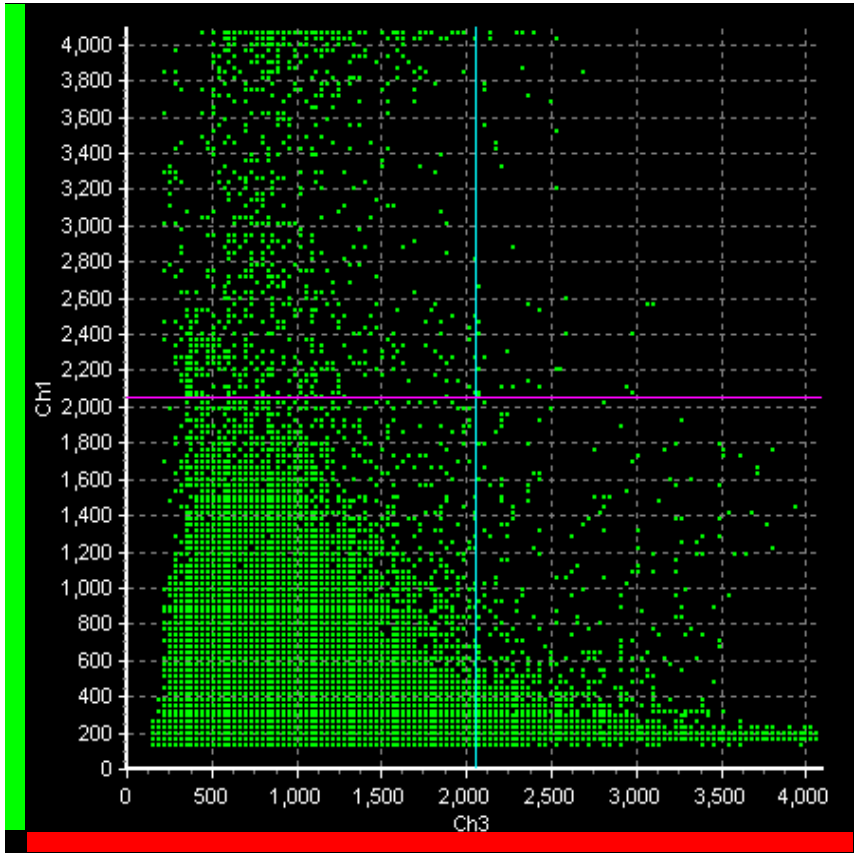


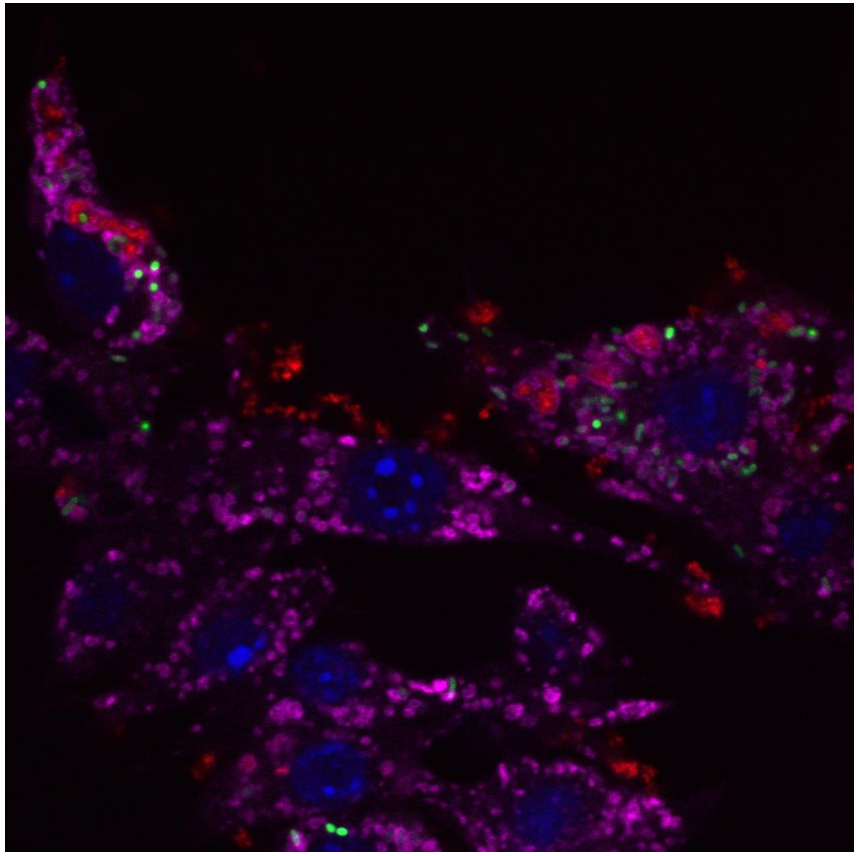


ROI	Area (pixels^2)	Pearson's Coeff.	Overlap	Overlap Index 1	Overlap Index 2	Coloc. Index 1	Coloc. Index 2
Entire Slice	1.0486E+06	0.43717	0.64569	0.88946	0.46873	0.12342	0.02395

Brucella melitensis in RAW 264.7 CELLS, 2 HRS P.T. (CC)

Colocalization of *B. melitensis (GFP)* and LAMP-1 *(Cy5)*

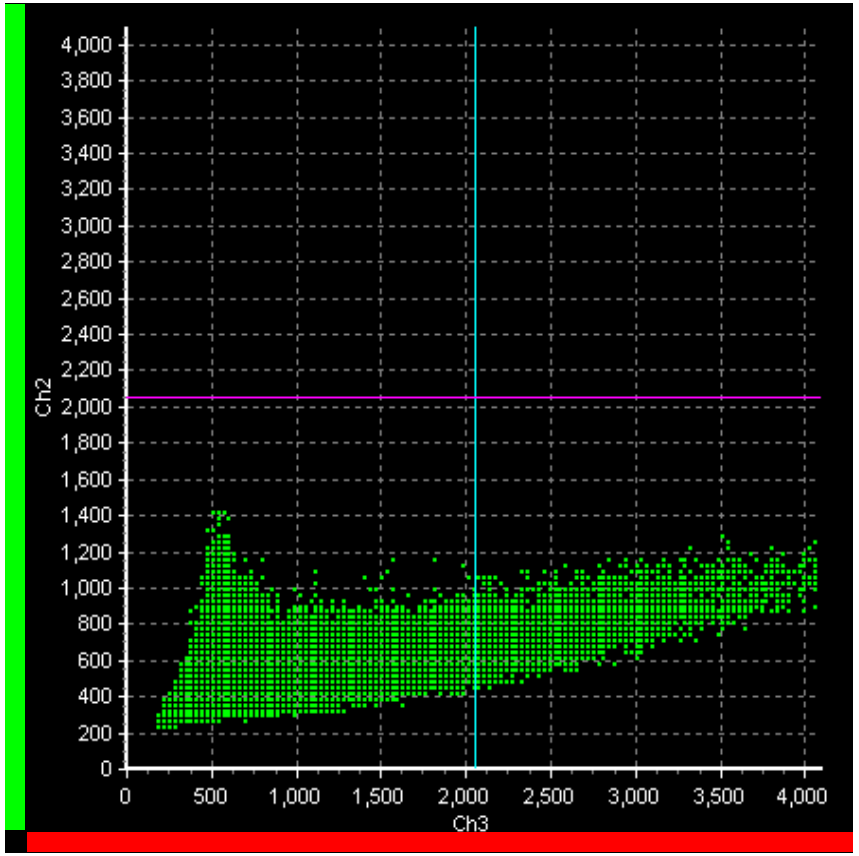


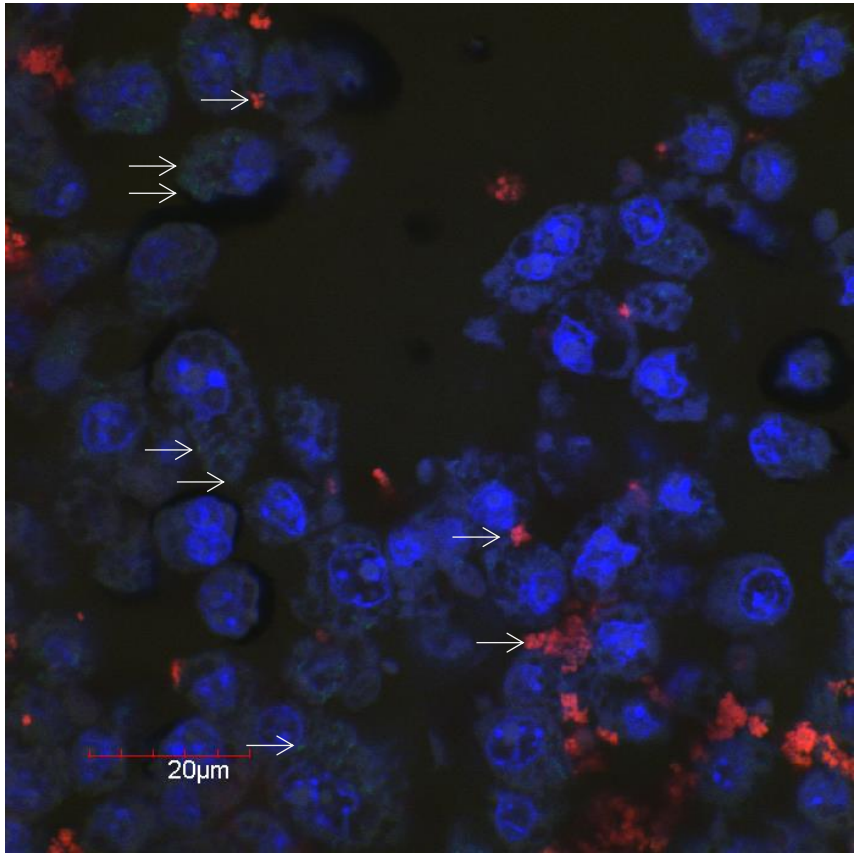


ROI	Area (pixels^2)	Pearson's Coeff.	Overlap	Overlap Index 1	Overlap Index 2	Coloc. Index 1	Coloc. Index 2
Entire Slice	1.0486E+06	0.19947	0.675457	0.29038	1.5725	0.0025612	0.011465

Brucella melitensis in RAW 264.7 CELLS, 24 HR P.T. (CC)

Colocalization of *B. melitensis (GFP)* and Nanoparticles with Antibiotic *(Rhod-B)*

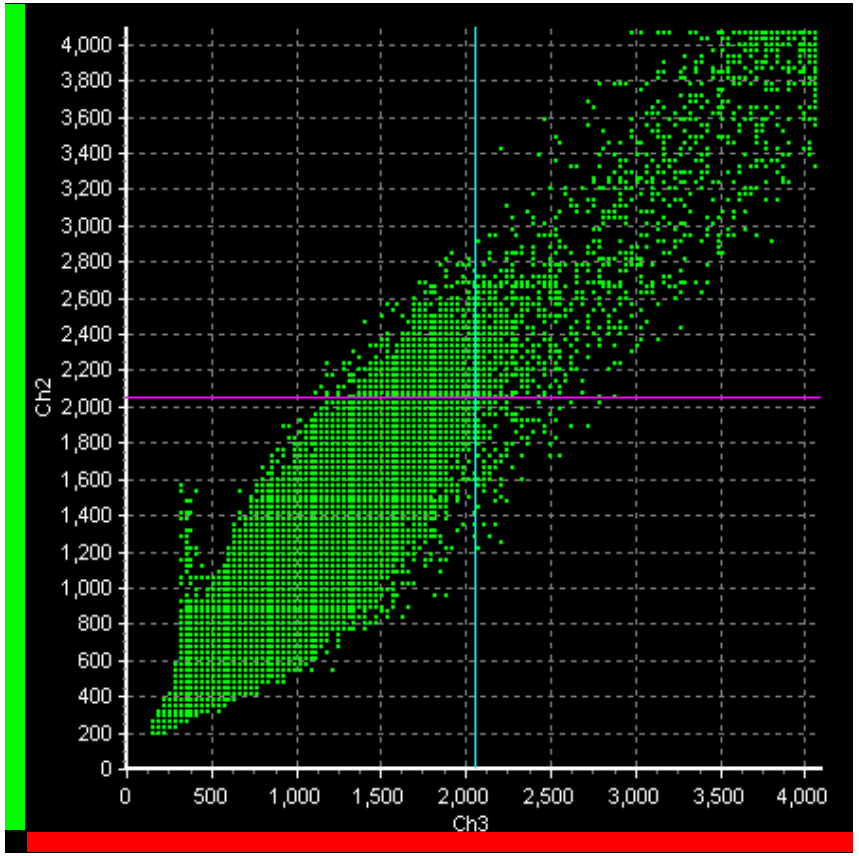


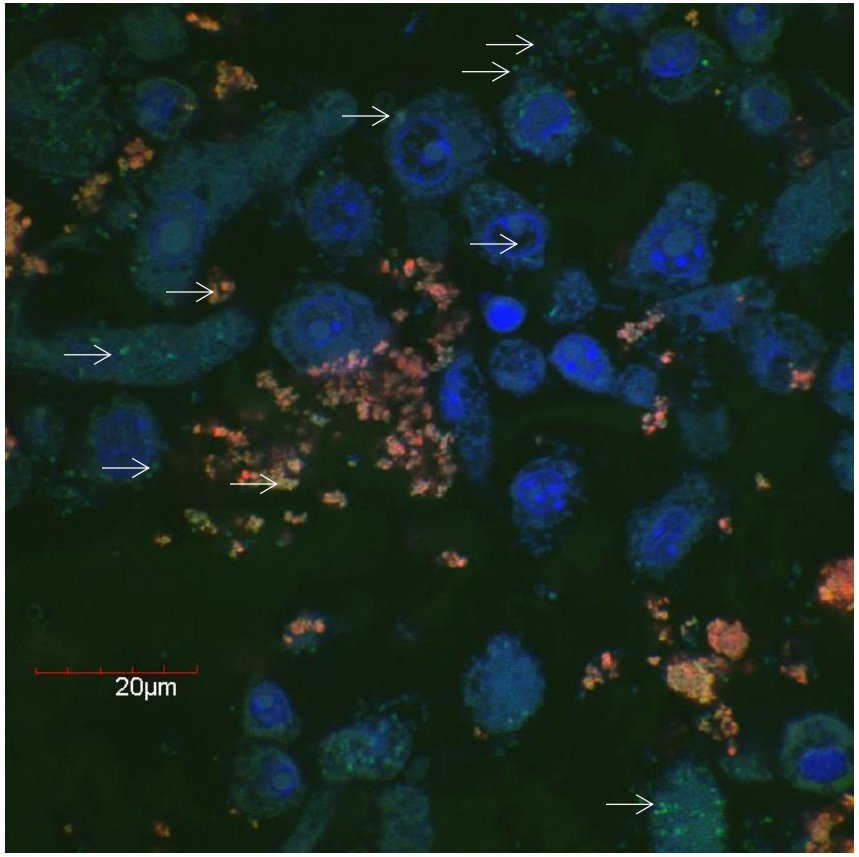


ROI	Area (pixels^2)	Pearson's Coeff.	Overlap	Overlap Index 1	Overlap Index 2	Coloc. Index 1	Coloc. Index 2
Entire Slice	6.40E+05	0.45195	0.93573	0.87632	0.99917	0	0.0099362

Brucella melitensis in RAW 264.7 CELLS, 24 HR P.T. (CCB)

Colocalization of *B. melitensis (GFP)* and Nanoparticles without Antibiotic *(Rhod-B)*

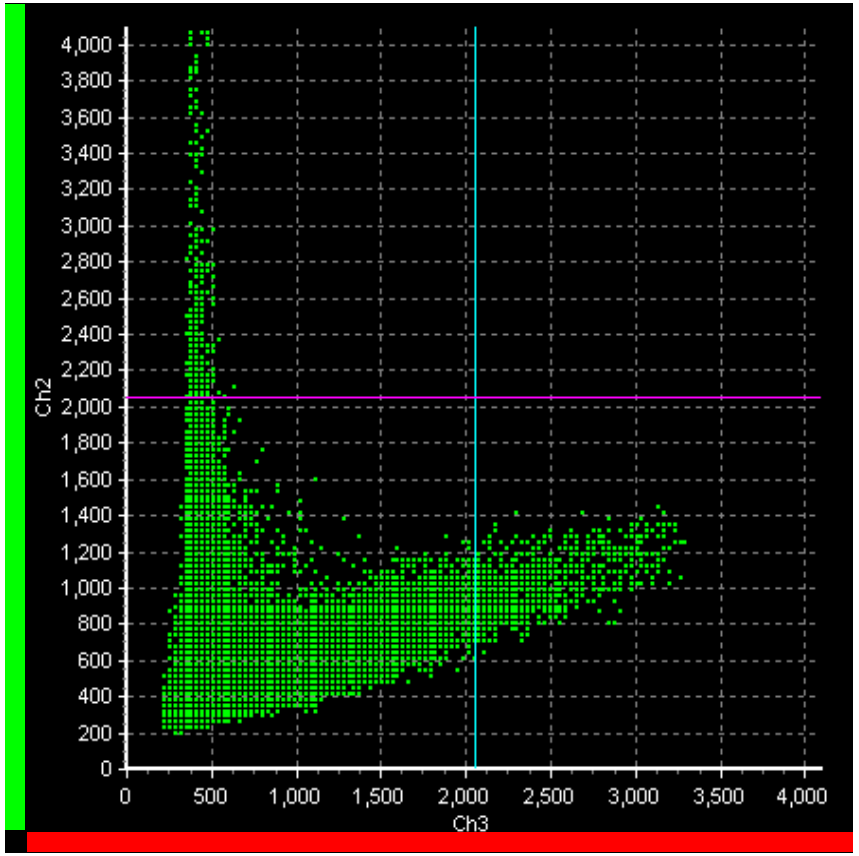


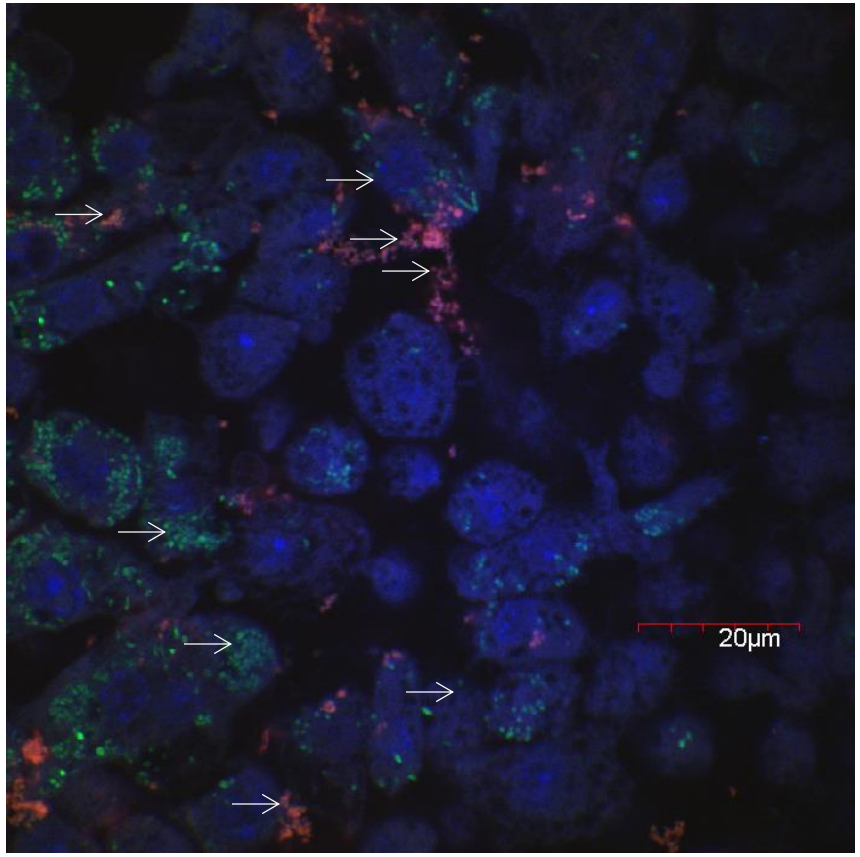


ROI	Area (pixels^2)	Pearson's Coeff.	Overlap	Overlap Index 1	Overlap Index 2	Coloc. Index 1	Coloc. Index 2
Entire Slice	6.40E+05	0.68038	0.86893	1.2357	0.61101	0.0062776	0.027314

Brucella melitensis in RAW 264.7 CELLS, 24 HR P.T. (CS)

Colocalization of *B. melitensis (GFP)* and Nanoparticles with Antibiotic *(Rhod-B)*

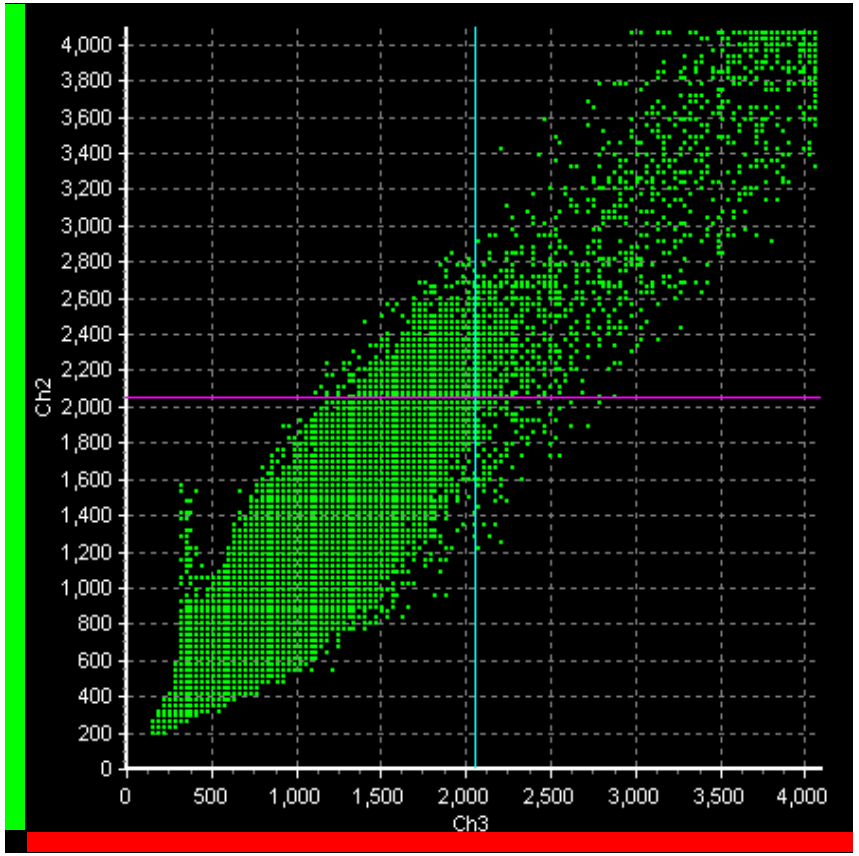


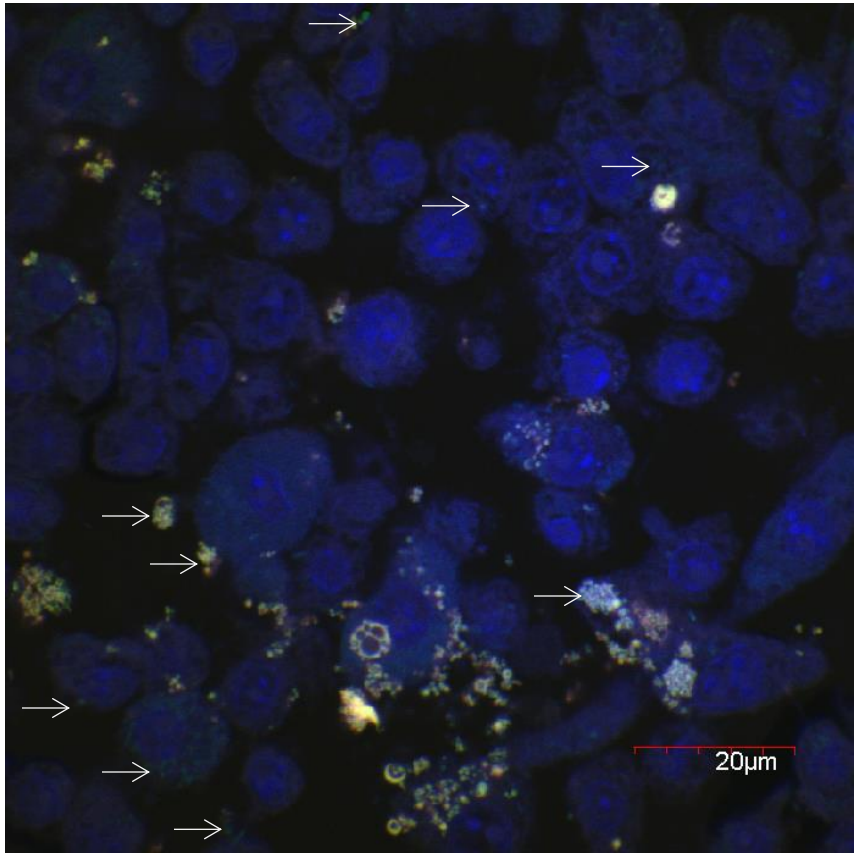


ROI	Area (pixels^2)	Pearson's Coeff.	Overlap	Overlap Index 1	Overlap Index 2	Coloc. Index 1	Coloc. Index 2
Entire Slice	6.40E+05	0.46532	0.92486	0.94313	0.90694	0.00066127	0.0043028

Brucella melitensis in RAW 264.7 CELLS, 24 HR P.T. (CSB)

Colocalization of *B. melitensis (GFP)* and Nanoparticles without Antibiotic *(Rhod-B)*





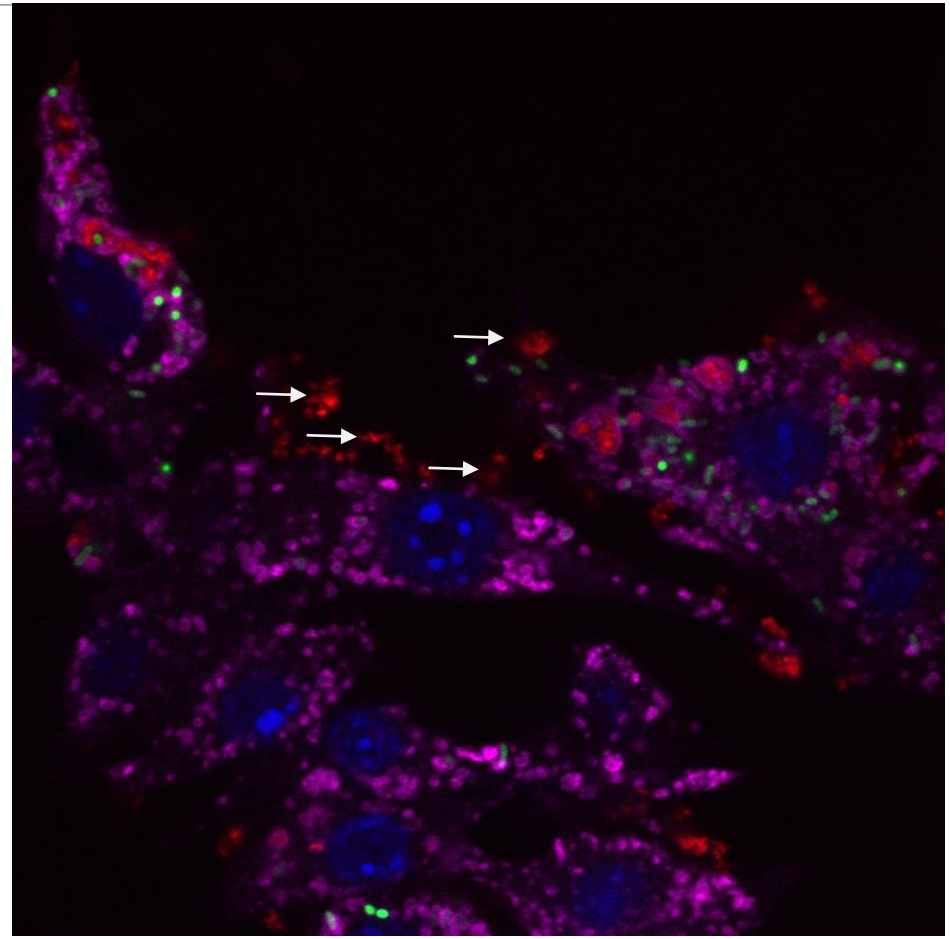
ROI	Area (pixels^2)	Pearson's Coeff.	Overlap	Overlap Index 1	Overlap Index 2	Coloc. Index 1	Coloc. Index 2
Entire Slice	6.40E+05	0.94981	0.98905	1.0748	0.91019	0.026637	0.017083

Results

- At 24 hours post treatment blank nanoparticles (CCB/CSB) had significantly larger Pearson Coefficients compared to (CC/CS)
- 20:80 CCB Pearson Coefficient Average (0.713)
- 20:80 CSB Pearson Coefficient Average (0.927)

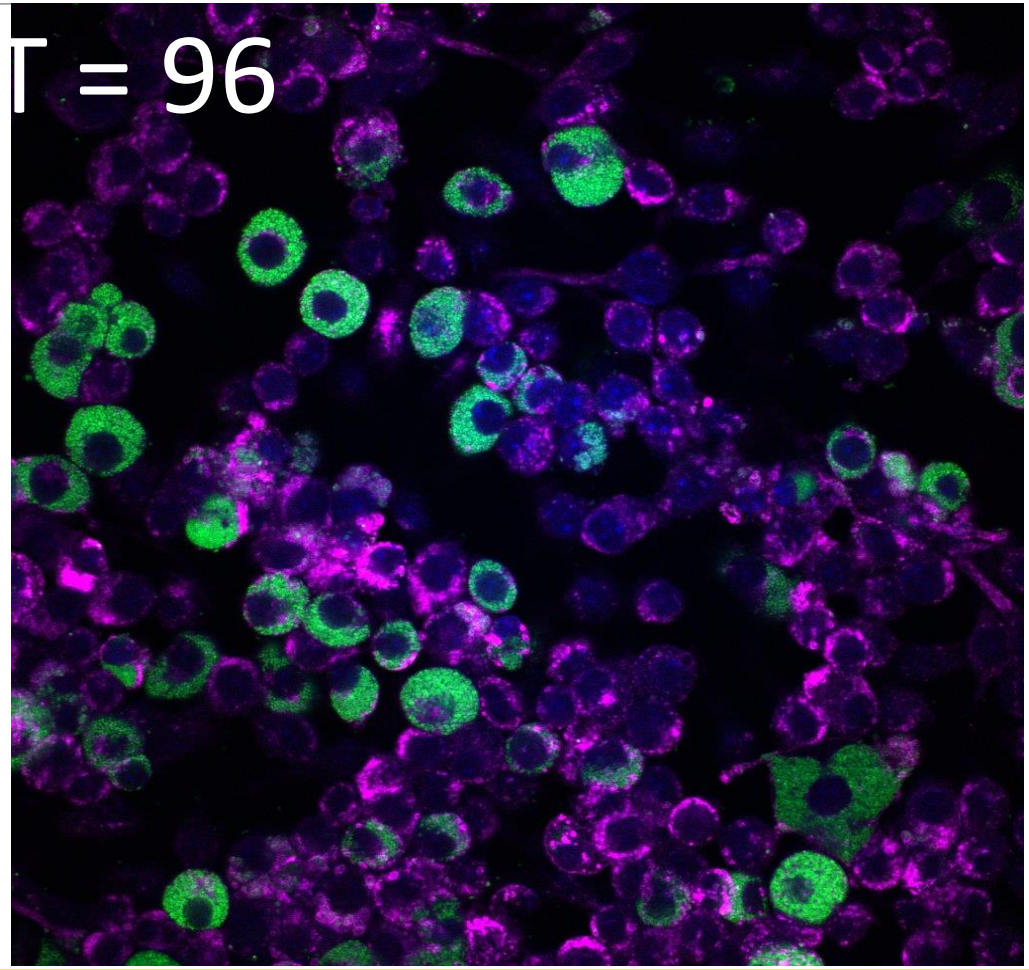
Key Observation

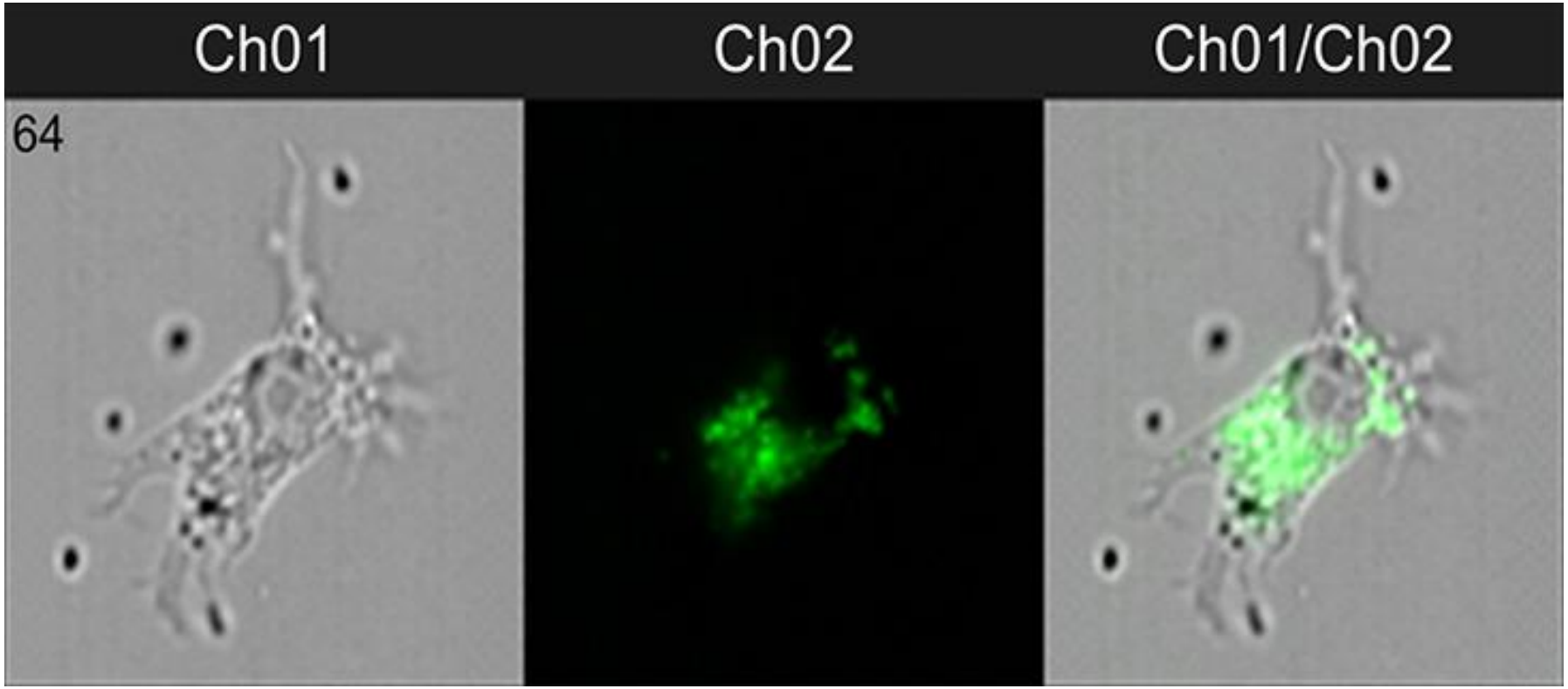
- Although nanoparticles were observed in phagosome, many were outside of cells, sometimes causing a lot of background in microscopy images



Key Observation

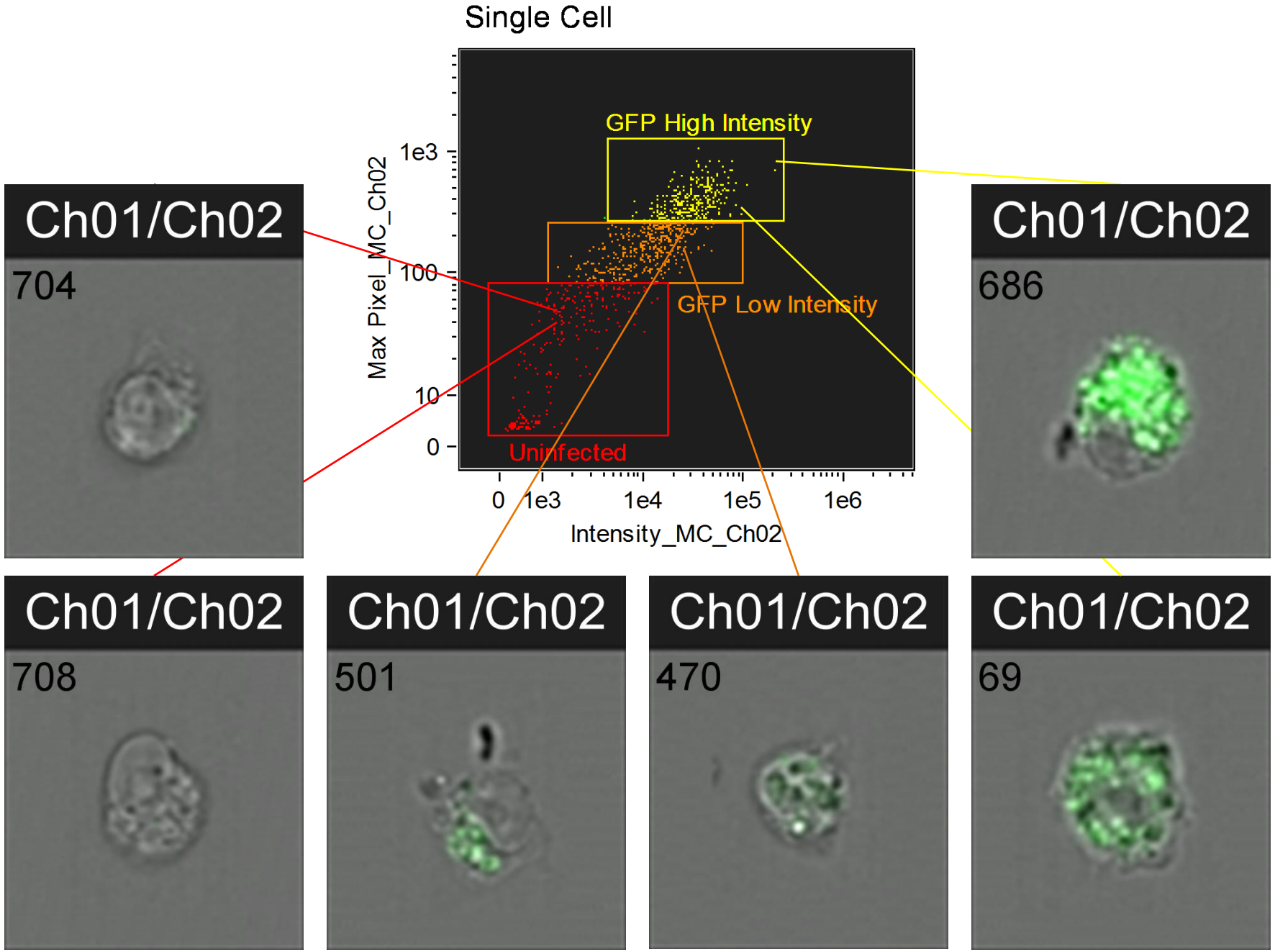
- With microscopy, there was difficulty quantifying monolayer sub-population (High-Low-No Infection)
- Difficulty gating populations





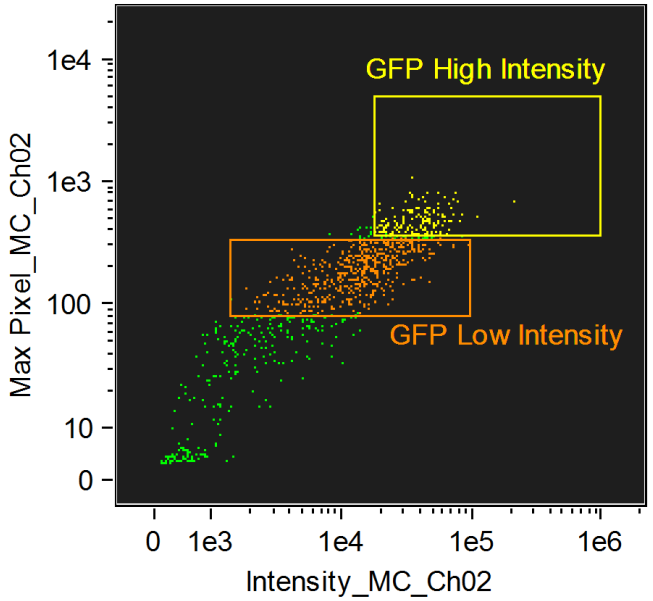
Imagestream

UTILIZING MULTI-SPECTRAL IMAGING FLOW CYTOMETRY TO QUANTIFY THE INTERNALIZATION/COLOCALIZATION OF POLYANHYDRIDE NANOPARTICLES OR BACTERIA IN RAW 264.7 CELLS


Single Cell
GFP High Intensity
GFP Low Intensity
Uninfected
Max Pixel_MC_Ch02
1e3
100
10
0
0
1e3
1e4
1e5
1e6
Intensity_MC_Ch02
Ch01/Ch02
704
Ch01/Ch02
686
Ch01/Ch02
708
Ch01/Ch02
501
Ch01/Ch02
470
Ch01/Ch02
69

UNTREATED

T = 0



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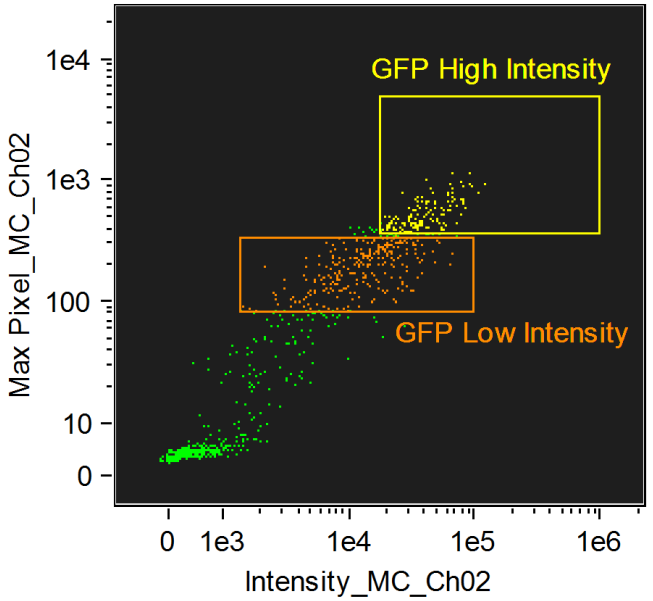
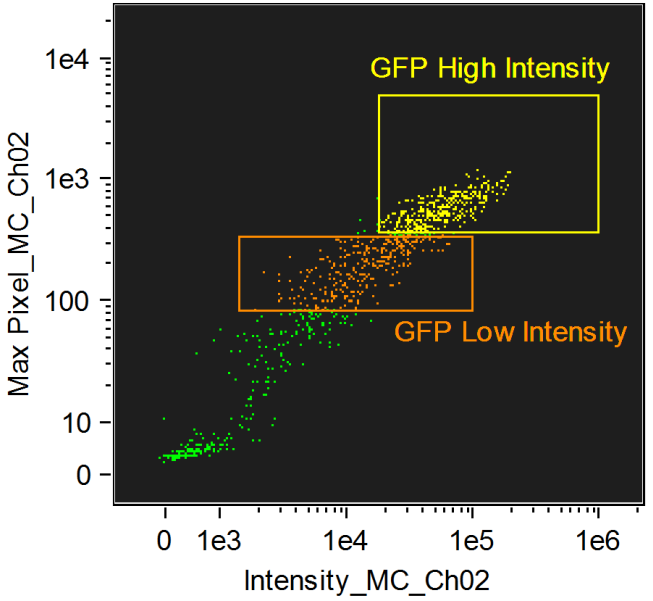
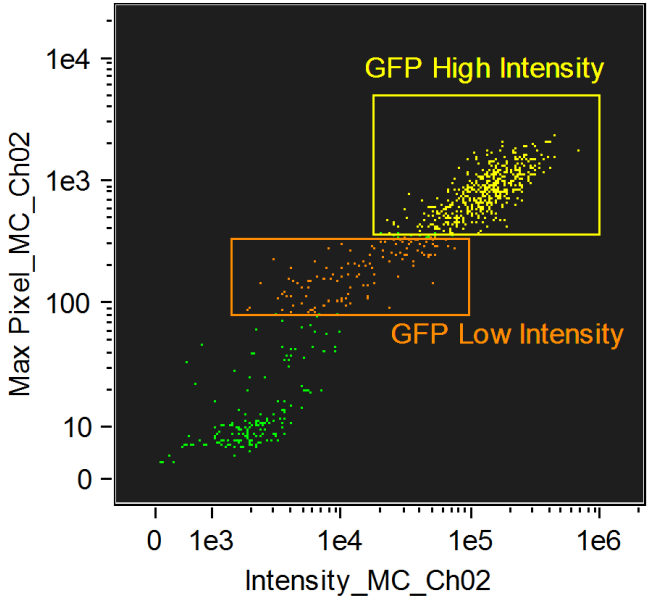


T = 48



T = 72



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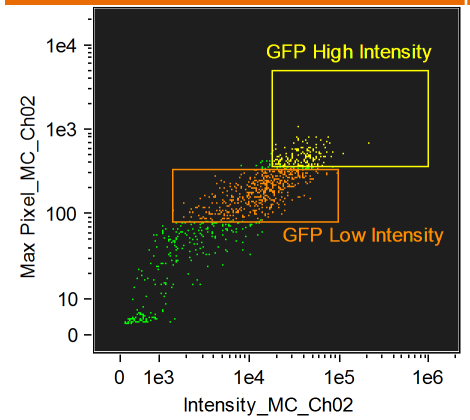
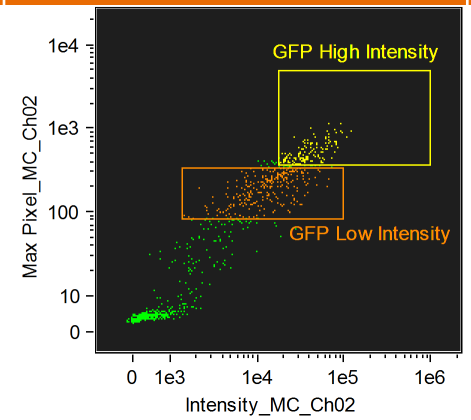


T = 0



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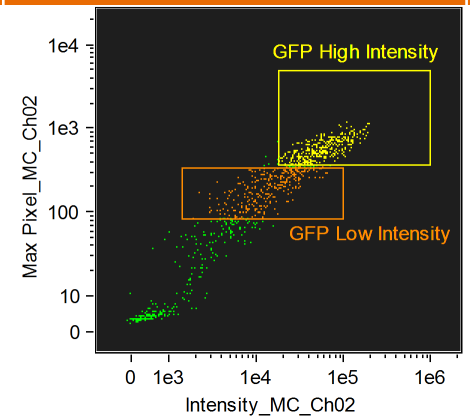

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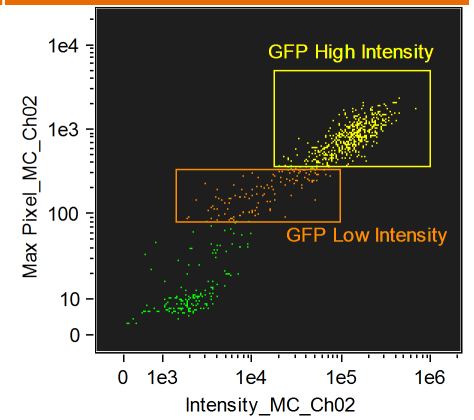

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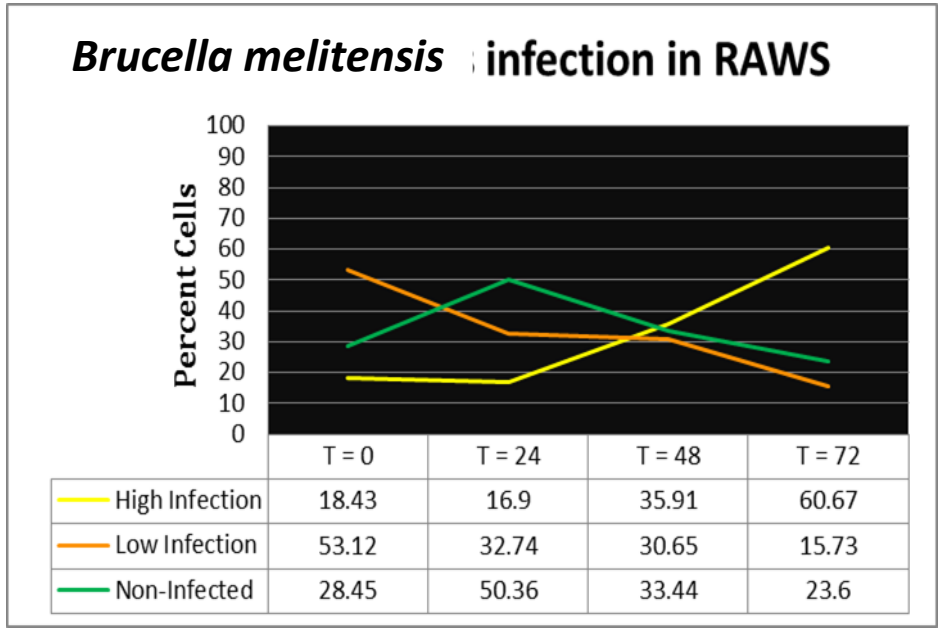
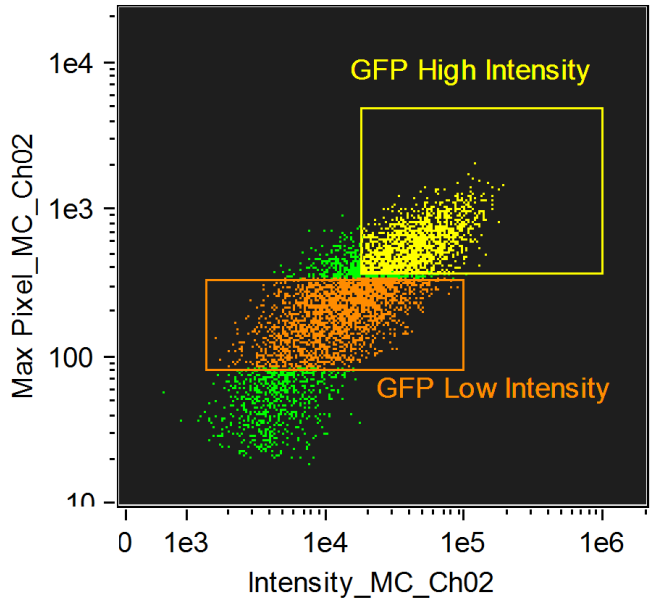


	T = 0	T = 24	T = 48	T = 72
High Infection	18.43	16.9	35.91	60.67
Low Infection	53.12	32.74	30.65	15.73
Non-Infected	28.45	50.36	33.44	23.6

UNTREATED

T = 0



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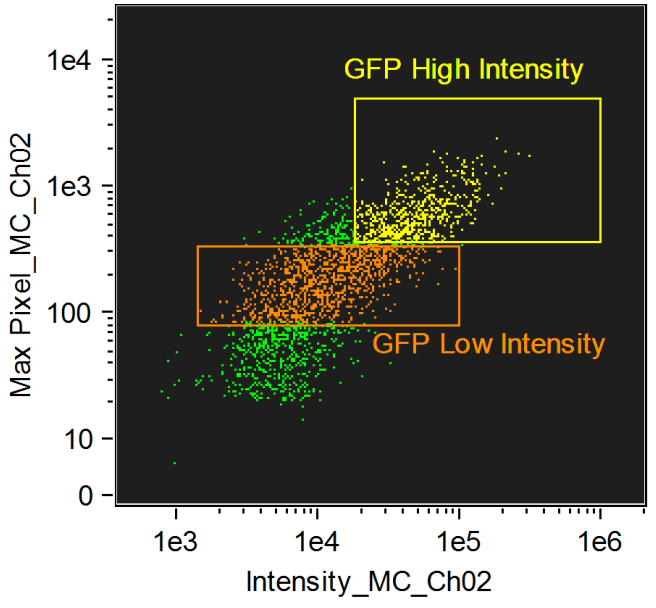

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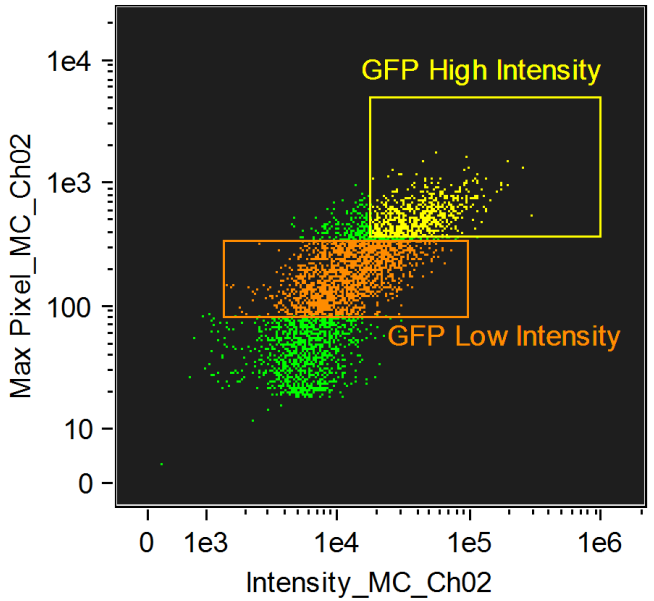

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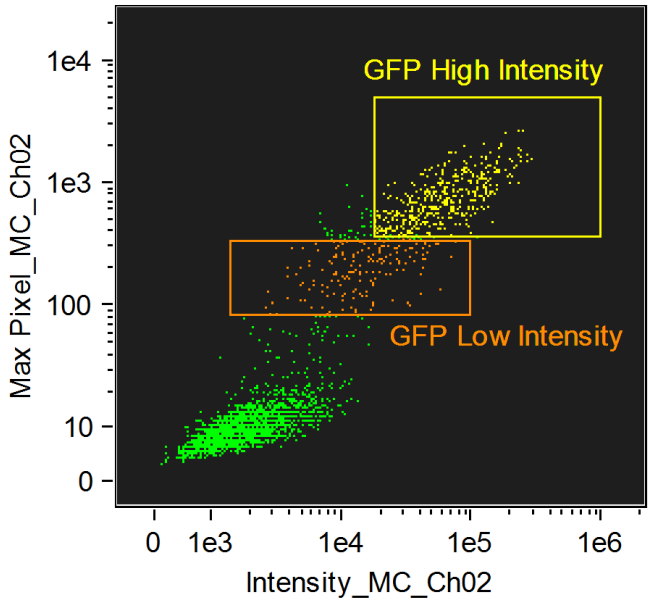
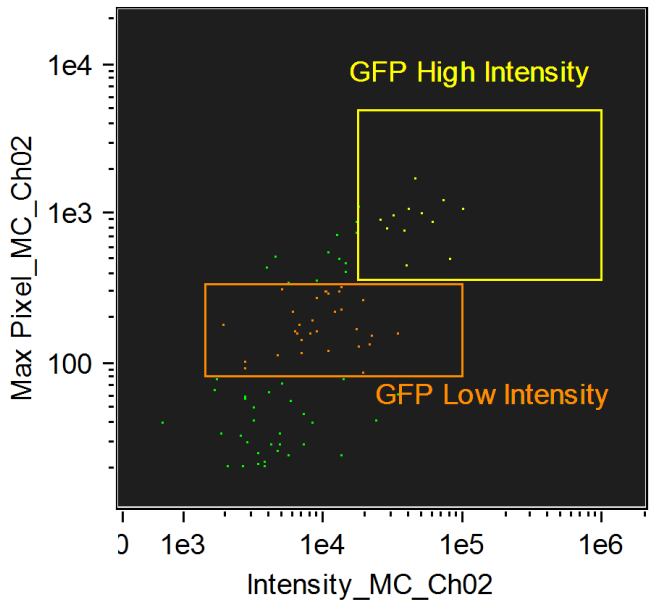
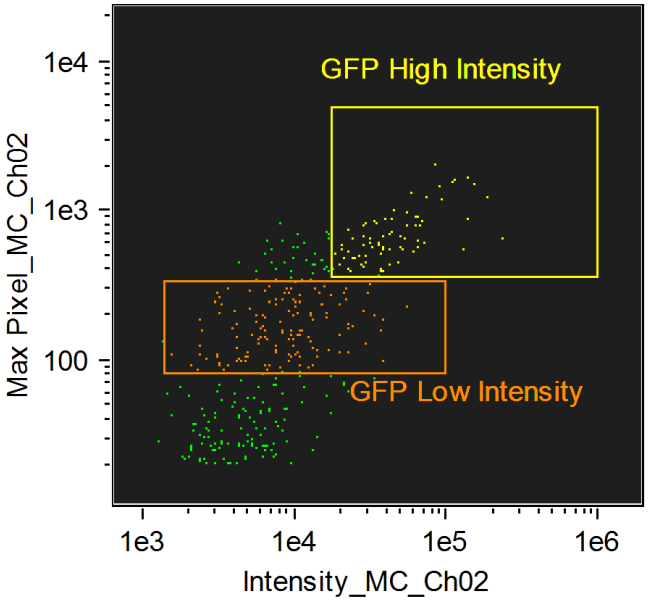


SOLUBLE

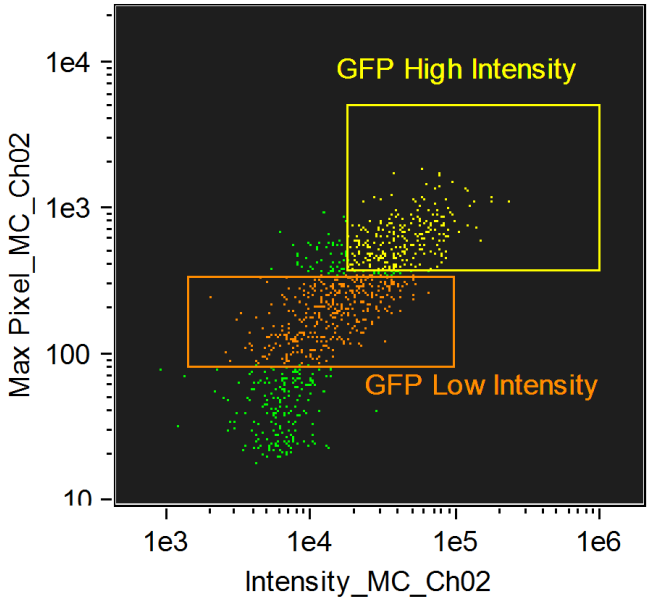
T = 0



T = 26



T = 48



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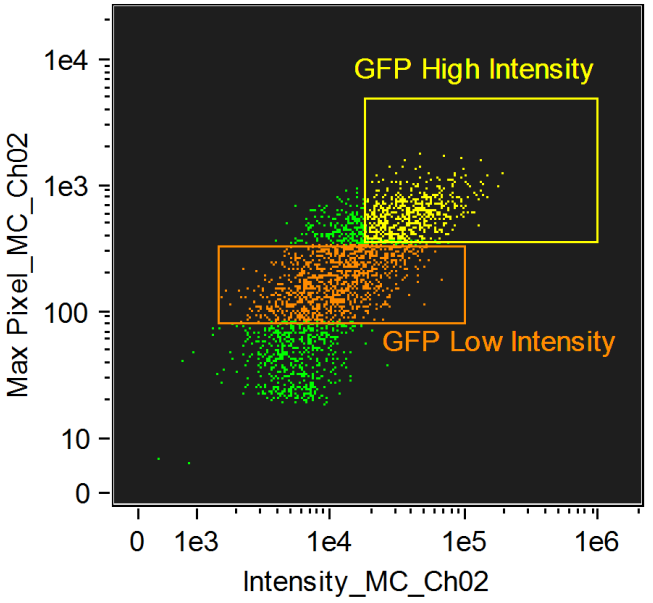

20:80 CPTEG:CPH

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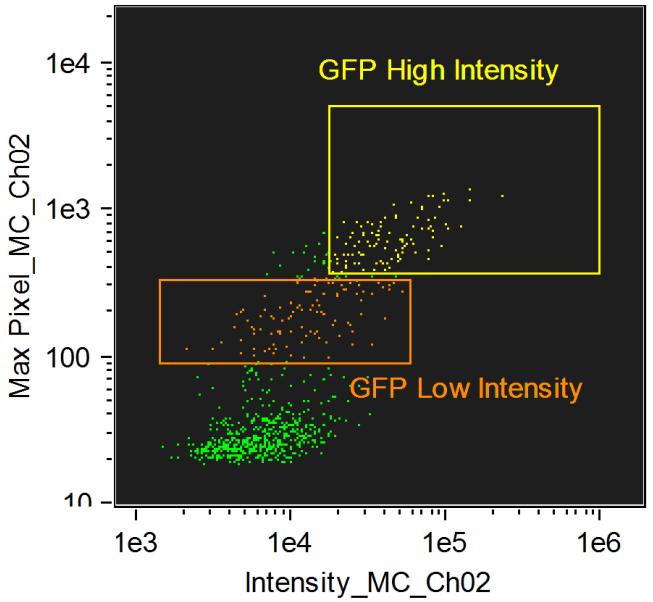

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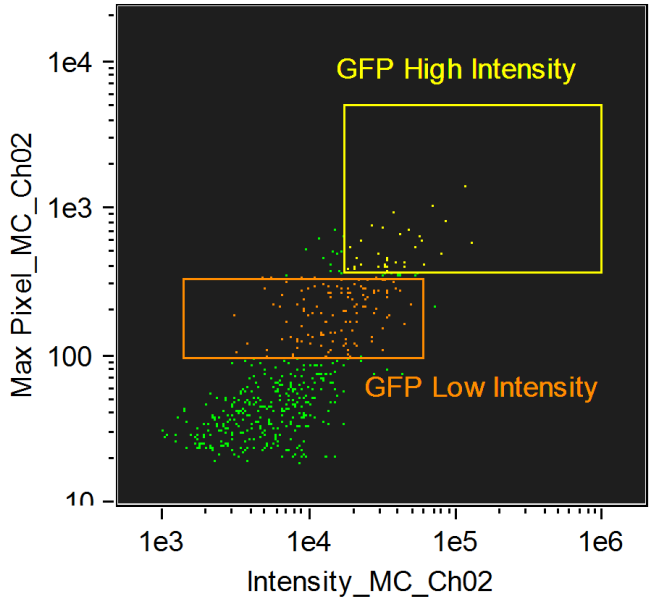

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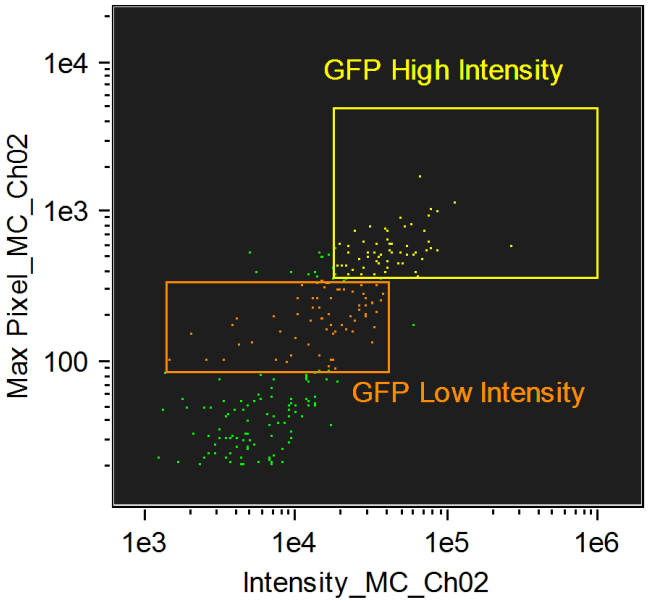

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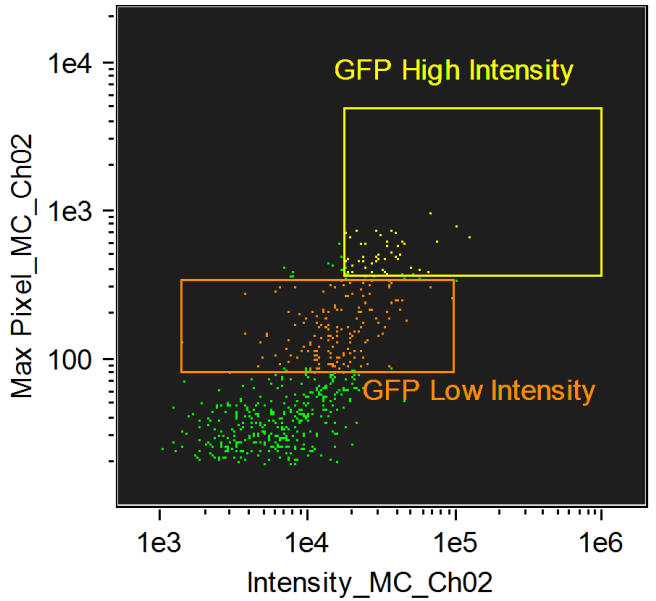


ImageStream Significance

IS capable of detecting both internalized nanoparticles as well as bacteria, and excluding external components

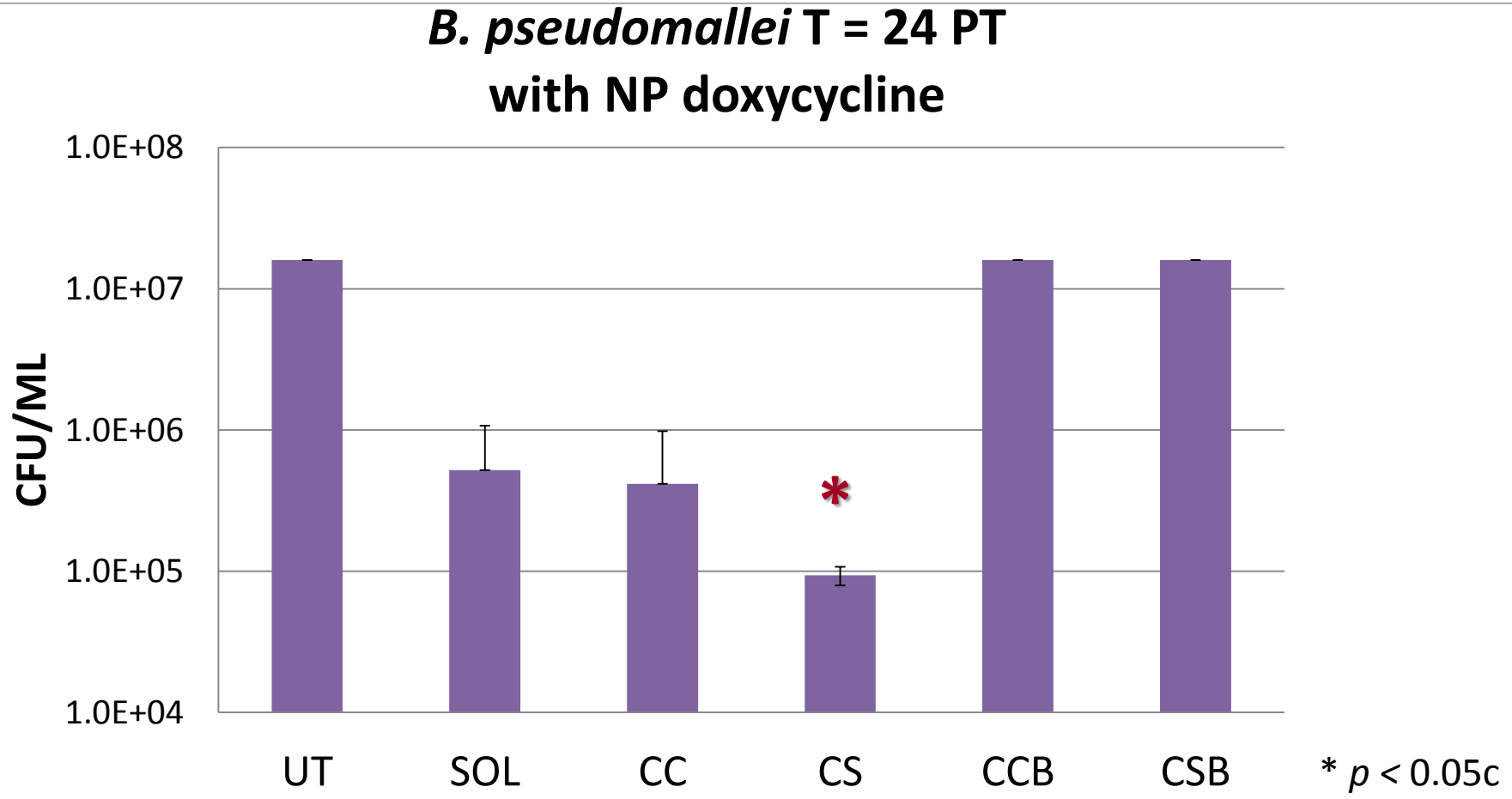
IS capable of gating and generating robust statistical data about subpopulations within an infection profile.

IS capability provides an accurate cell-by-cell analysis of fluorescent signal intensities and spatial relationships (colocalization) between different structures and cellular features at high speed.

Conclusions

- Encapsulated PA nanoparticles are effective therapeutic agents capable of reducing/eliminating bacterial load in murine macrophages.
  - PA nanoparticles localize in phagosomal compartments
  - PA nanoparticles safely deliver antigen
  - PA nanoparticles can control release of antigen
  - PA nanoparticles chemistry modifications alter therapeutic profile

Future Directions: *Burkholderia* spp.



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- God, Family (Wife-Patience), Kids, (Lucas, Manny, Amara, Moses-Michae, Yaa'El)
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